

POLYAMINES AS REGULATORS OF CELL GROWTH AND DIFFERENTIATION

POLYAMINE SYNTHESIS INHIBITORS AS ANTICANCER AGENTS

Stina M Oredsson



Lund 1984



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Lund 1984

*"You have changed my sadness into a joyful dance,
you have taken off my clothes of mourning,
and you have given me clothes of joy.
So I will not be silent.
I will sing praise to you.
Lord, you are my God,
I will give thanks to you forever."*

Psalms 30

To Sven

The present thesis is based on the following papers:

- I. Oredsson S, Anehus S and Heby O.
Irreversible Inhibition of the Early Increase in Ornithine Decarboxylase Activity Following Growth Stimulation is Required to Block Ehrlich Ascites Tumor Cell Proliferation in Culture. *Biochem. Biophys. Res. Commun.*, 94:151-158, 1980.
- II. Oredsson SM, Anehus S and Heby O.
Reversal of the Growth Inhibitory Effect of α -Difluoromethylornithine by Putrescine but not by Other Divalent Cations. Submitted for publication.
- III. Oredsson SM, Kanje M, Mamont PS, Wagner J and Heby O.
Polyamine Depletion Increases Cellular Ribonucleotide Levels. Submitted for publication.
- IV. Oredsson SM, Friend DS and Marton LJ.
Changes in Mitochondrial Structure and Function in 9L Rat Brain Tumor Cells Treated *In Vitro* with α -Difluoromethylornithine, a Polyamine Biosynthesis Inhibitor. *Proc. Natl. Acad. Sci. USA*, 80:780-784, 1983.
- V. Oredsson SM, Marton LJ, Billgren M and Heby O.
Induction of F9 Embryonal Carcinoma Cell Differentiation by Inhibition of Polyamine Synthesis. Submitted for publication.
- VI. Oredsson SM, Deen DF and Marton LJ.
Influence of Polyamine Depletion Caused by α -Difluoromethylornithine, an Enzyme-activated Irreversible Inhibitor of Ornithine Decarboxylase, on Alkylation- and Carbamoylation-induced Cytotoxicity in 9L Rat Brain Tumor Cells *In Vitro*. *Cancer Res.*, 43:4606-4609, 1983.
- VII. Oredsson SM, Deen DF and Marton LJ.
Decreased Cytotoxicity of *cis*-Diamminedichloroplatinum(II) by α -Difluoromethylornithine Depletion of Polyamines in 9L Rat Brain Tumor Cells *In Vitro*. *Cancer Res.*, 42:1296-1299, 1982.
- VIII. Oredsson SM, Gray JW, Deen DF and Marton LJ.
Decreased Cytotoxicity of 1- β -D-Arabinofuranosylcytosine in 9L Rat Brain Tumor Cells Pretreated with α -Difluoromethylornithine *In Vitro*. *Cancer Res.*, 43:2541-2544, 1983.

These papers will be referred to by their Roman numerals.

CONTENTS

1	ABBREVIATIONS	6
2	BACKGROUND	7
2.1	Early History of the Polyamines	7
2.2	Polyamine Structure	7
2.3	Occurrence of Polyamines	8
2.3.1	Polyamines in Viruses and Bacteriophages	8
2.3.2	Polyamines in Prokaryotic Cells	9
2.3.3	Polyamines in Eukaryotic Cells	9
2.4	Polyamine Synthesis	10
2.4.1	Ornithine Metabolism	10
2.4.2	Putrescine Synthesis	12
2.4.3	Spermidine and Spermine Synthesis	14
2.4.4	Salvage Pathway of Adenine Nucleotide Synthesis	17
2.4.5	Salvage Pathway of Methionine Synthesis	18
2.5	Polyamine Synthesis Inhibitors	18
2.5.1	Ornithine Decarboxylase Inhibitors	18
2.5.2	S-Adenosylmethionine Decarboxylase Inhibitors	21
2.6	Polyamines as Regulators of Cell Growth	22
2.6.1	Polyamines in Proliferating Cells	22
2.6.2	Polyamine Synthesis Inhibition and Its Effects on DNA Replication	24
2.7	Polyamines as Regulators of Cell Differentiation	26
2.8	Polyamine Synthesis Inhibitors as Anticancer Agents	27
2.8.1	The Stabilizing Effects of Polyamines on DNA	27
2.8.2	Cell Cycle Specific Block by Polyamine Synthesis Inhibition	30
3	THE PRESENT STUDY	30
3.1	Specific Aims	30
3.2	Polyamines as Regulators of Cell Growth - Experimental Systems	32
3.2.1	Ehrlich Ascites Tumor Cells	32
3.2.2	Rat Brain Tumor Cells	33
3.3	Polyamines as Regulators of Cell Differentiation - Experimental System	33
3.3.1	Embryonal Carcinoma Cells	34
3.4	Polyamine Synthesis Inhibitors as Anticancer Agents - Experimental System	34
3.4.1	Rat Brain Tumor Cells	34

3.5	Preparative and Analytical Procedures	35
3.5.1	Collection and Counting of Cells	35
3.5.2	Polyamine Analysis	35
3.5.3	Ornithine Decarboxylase Assay	36
3.5.4	Cell Preparative Procedures for Flow Cytometric Analysis	37
3.5.5	Cell Kinetic Analysis by Flow Cytometry	38
3.5.6	S-Adenosylmethionine and Decarboxylated S-Adenosylmethionine Analysis	39
3.5.7	Ribonucleotide Analysis	39
3.5.8	Deoxyribonucleotide Analysis	39
3.5.9	Cell Preparative Procedures for Electron Microscopy	40
3.5.10	Pyruvate Utilization Analysis	40
3.5.11	Plasminogen Activator Assay	41
3.5.12	Colony Forming Efficiency Assay	42
3.6	Polyamines as Regulators of Cell Growth - Results and Discussion	43
3.6.1	Effects of Polyamine Depletion on Cell Growth	43
3.6.2	Reversal of DFMO-Induced Growth Inhibition	47
3.6.3	Effects of DFMO Treatment on Cellular Ribonucleotide and Deoxyribonucleotide Levels	50
3.6.4	Ultrastructural Changes in DFMO-Treated Cells	55
3.7	Polyamines as Regulators of Cell Differentiation - Results and Discussion	57
3.8	Polyamine Synthesis Inhibitors as Anticancer Agents - Results and Discussion	60
3.8.1	Effects of DFMO-Induced Polyamine Depletion on Alkylation- and Carbamoylation-Induced Cytotoxicity	60
3.8.2	Effects of DFMO-Induced Polyamine Depletion on the Cytotoxicity of <i>cis</i> -Diamminedichloroplatinum(II)	64
3.8.3	Effects of DFMO-Induced Polyamine Depletion on the Cytotoxicity of an S phase Specific Agent	66
3.9	Summary	67
4	ACKNOWLEDGEMENTS	70
5	REFERENCES	73

1. ABBREVIATIONS

ara-C	1- β -D-arabinofuranosylcytosine
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BHCNU	1,3-bis(<i>trans</i> -4-hydroxycyclohexyl)-1-nitrosourea
chlorozotocin	2-(3-(2-chloroethyl)-3-nitrosoureido)-D-glucopyranose
<i>cis</i> -platinum	<i>cis</i> -diamminedichloroplatinum(II)
deSAM	decarboxylated S-adenosylmethionine
DFMO	α -difluoromethylornithine
ELD	Ehrlich Lettré Diploid ascites tumor cells
ENU	N-ethyl-N-nitrosourea
MGBG	methylglyoxal-bis(guanylhydrazone)
MNU	N-methyl-N-nitrosourea
MO	α -methylornithine
MTA	5'-methylthioadenosine
ODC	ornithine decarboxylase
PBS	phosphate-buffered saline
PCA	perchloric acid
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase

2. BACKGROUND

2.1 EARLY HISTORY OF THE POLYAMINES

In 1678, the Dutch microscope inventor and researcher Antonie van Leeuwenhoek, for the first time described human spermatozoa in his famous letter to the Royal Society in London (1). In this letter he also reported that continuous observation of a single specimen of semen in a microscope permitted him to study the gradual formation of colorless crystals.

Subsequently, similar crystals were described by Vauquelin (2), Charcot and Robin (3), Boettcher (4), Schreiner (5) and several other investigators. The crystals were detected in human semen and other body fluids, as well as in tissue specimens, particularly after storage. Various names were applied to them and finally in 1888 they were named "spermine" by Ladenburg and Abel (6). The chemical nature of the crystals, however, remained unclear for another half century until the famous work of Otto Rosenheim. In 1924 to 1926 Rosenheim and his collaborators isolated spermine from various mammalian tissues and confirmed the structure by synthesis (7, 8). In addition they isolated a related compound, which they named "spermidine" (9). Still another related compound was isolated from decomposing animal material. It was named "putrescine", derived from the word putrefaction, because of its origin and foul smell.

Thus began the history of the three most common natural polyamines about two hundred years before that of the nucleic acids. Regarding the crystals described by Leeuwenhoek (1), we know today that the slow deposition of spermine phosphate requires a prior liberation of inorganic phosphate via the enzymatic hydrolysis of the phosphorylcholine present in seminal fluid. Spermine phosphate crystallizes easily and spontaneously.

2.2 POLYAMINE STRUCTURE

Subsequent studies have shown that the aliphatic polyamines putrescine, spermidine and spermine are widely distributed in nature. The structures of these nonprotein nitrogenous bases are shown in Figure 1. Under physiological conditions the primary and secondary amino groups are protonated thus giving the polyamines 2-4 positive charges. Rotations around the carbon-carbon and carbon-nitrogen bonds

impart considerable conformational flexibility of these polycations. In contrast to inorganic cations, which are derived from the circulation, polyamines are synthesized in the cell by enzymatic reactions regulated by many factors.

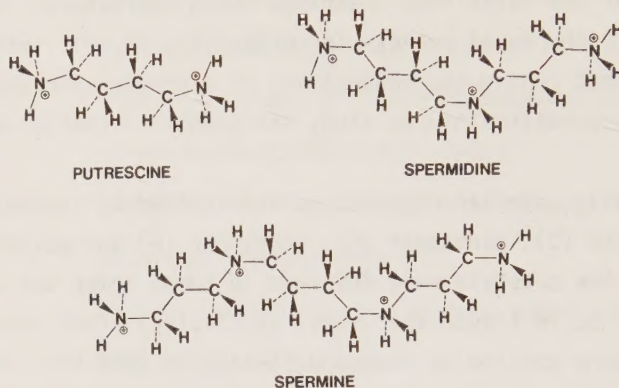


Figure 1. The molecular structures of the three major polyamines present in mammalian cells. At a physiological pH the primary and secondary amino groups are protonated.

2.3 OCCURRENCE OF POLYAMINES

2.3.1 Polyamines in Viruses and Bacteriophages

Polyamines have been found in several viruses and bacteriophages, where they may occur as major cationic constituents (10). Although there are several studies showing that the production of viruses (11-14) and bacteriophages (10, 15) is impaired in polyamine-depleted cells, the significance of polyamines for viral function and multiplication is still not understood in detail. It has been proposed that the polyamines present in viruses and bacteriophages are involved in the neutralization of the negatively charged phosphate groups of the nucleic acids, and that they are required for the extremely tight packaging of the DNA or RNA inside the virus and bacteriophage particles (16). Other possible roles of the polyamines include the maintenance of the genome in a particular configuration for the purpose of transcription and translation (17). Despite the lack of a clearly defined role of the polyamines in viruses, their selective incorporation into virions suggests that they may serve an important function in the virus life cycle (17).

2.3.2 Polyamines in Prokaryotic Cells

The finding of Herbst and Snell (18), that polyamines were required for growth of *Hemophilus parainfluenzae*, is the earliest demonstration of an essential biological role for polyamines. These studies showed that when the bacteria were seeded into a medium lacking polyamines they did not grow.

Subsequent studies have shown that polyamines are growth factors for several bacterial strains (19, 20). The isolation of mutants of *Escherichia coli* with specific defects in the various steps of polyamine synthesis, has greatly facilitated the analysis of the physiological function of the polyamines in prokaryotes. Data derived from studies of polyamine-deficient bacterial mutants suggests that polyamines are necessary for the maintenance of an optimal growth rate.

Interestingly, prokaryotic cells normally contain only putrescine and spermidine in contrast to eukaryotic cells that contain all three polyamines (20, 21). The enzyme catalyzing the synthesis of spermine has not been detected in most prokaryotic cells. Nevertheless, when spermine is present in the growth medium of bacteria it is readily taken up, and it has been observed to stimulate growth (19). Interestingly, Paulin et al. (22) recently presented the first evidence of a spermine synthesizing enzyme in cell extracts of a prokaryote, *Acetobacteria*. A number of unusual polyamines have been discovered in thermophilic bacteria and it has been suggested that they are necessary for thermostability (23-26).

2.3.3 Polyamines in Eukaryotic Cells

Putrescine, spermidine and spermine are widely distributed in eukaryotic cells. Changes in their concentrations have been associated with a number of important cell activities.

A variety of invertebrates such as polychaetes, sea urchins, nudibranchs and the slime mold have been used in studies of the physiological role of the polyamines (27, 28). Polyamines appear to be vital for the infectivity of parasites causing diseases such as sleeping sickness, malaria and coccidiosis (29). In recent years, studies of polyamines in lower and higher plants have received increasing interest (30). However, the majority of studies on eukaryotes have been performed with various mammalian systems (31).

It has invariably been demonstrated that the activities of the

polyamine biosynthetic enzymes and the concentrations of the polyamines increase precipitously when growth and differentiation is induced. Stimulation of polyamine synthesis appears to precede initiation of DNA synthesis, indicating the possibility of polyamines being involved in the regulation of this event. Despite a large body of information implicating the polyamines in the regulation of cell growth and differentiation their function at the molecular level in the cell is still not known in detail.

2.4 POLYAMINE SYNTHESIS

2.4.1 Ornithine Metabolism (Fig. 2)

The decarboxylation of L-ornithine (an amino acid that is not part of proteins), catalyzed by ornithine decarboxylase (ODC) (L-ornithine carboxy-lyase; EC 4.1.1.17) may be considered the initial step in mammalian polyamine synthesis.

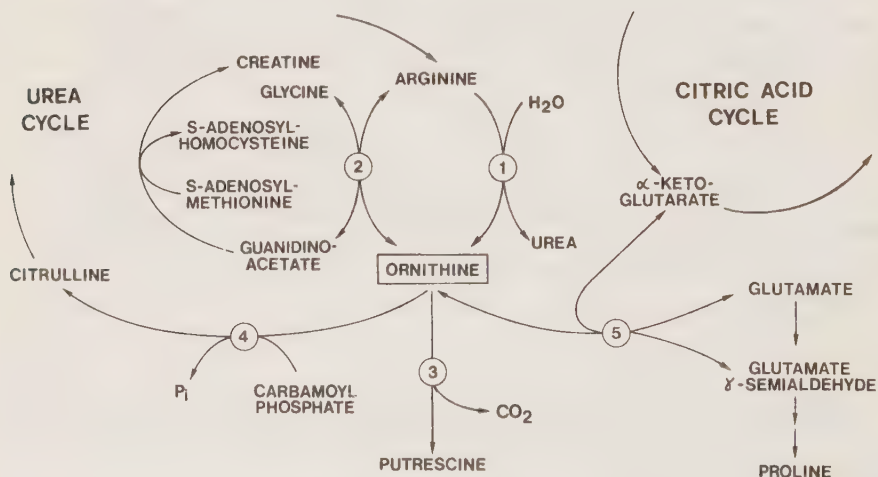


Figure 2. Ornithine metabolism in mammalian cells. Two enzymes are involved in ornithine synthesis: 1) arginase and 2) transaminase. Both use arginine as a substrate. Ornithine serves as a substrate in three major pathways. 3) Ornithine decarboxylase catalyzes the irreversible formation of putrescine, the precursor of spermidine and spermine. 4) Ornithine carbamoyltransferase catalyzes the second step in the urea cycle, which is irreversible because of the high transfer potential of carbamoyl phosphate. 5) Ornithine aminotransferase catalyzes the first step in the ornithine-proline pathway, which is potentially reversible. It plays an important role in regulating the amount of ornithine entering the urea cycle and in transferring intermediates between the urea and citric acid cycles. Ornithine may also serve as a substrate in the transaminase-catalyzed reaction, because this reaction is readily reversible.

A number of enzymes are involved in the metabolism of ornithine. One of the enzymes is arginase (L-arginine amidinohydrolase; EC 3.5.3.1), which catalyzes the hydrolysis of arginine to ornithine and urea. This enzyme was originally detected in mammalian liver and is the terminal enzyme of the urea cycle (32, 33). Arginase activity also occurs in tissues which are devoid of a complete urea cycle (34-36). In such cells arginase may be regarded as the initial event in putrescine synthesis. Indeed, an elevation of the arginase activity has been shown to accompany an elevated polyamine biosynthesis in many tissues, such as androgen-stimulated prostate (37), hormonally stimulated mammary explants (38), insulin-stimulated chicken liver (39), concanavalin A-activated lymphocytes (40) and hyperplastic epidermis (41). Pohjanpelto and Hölttä (42) found that the arginase activity was generally higher than that of ODC and that there was no correlation between the two activities in a number of different cell lines.

Ornithine is also formed in a reaction catalyzed by transamidinase (L-arginine-glycine amidinotransferase; EC 2.1.4.1) where arginine and glycine serve as substrates. Guanidinoacetate is the other product of transamidinase action and a substrate in creatine synthesis. In cells that do not have a complete urea cycle and are not dependent on creatine synthesis the primary function of arginase and transamidinase might be to channel arginine into polyamine synthesis.

In addition to ODC there are at least two other enzymes in mammalian cells that use ornithine as a substrate. One is ornithine carbamoyltransferase (carbamoyl phosphate:L-ornithine carbamoyltransferase; EC 2.1.3.3), a urea cycle enzyme that catalyzes the transfer of a carbamoyl group to ornithine, thus forming citrulline. This enzyme is localized in the mitochondrial matrix and ornithine uptake by the mitochondria is mediated by a specific transport system (43). A significant activity of ornithine carbamoyltransferase is only found in the liver, where it constitutes an important step in the urea cycle (44). The other enzyme, ornithine aminotransferase (L-ornithine:2-oxoacid aminotransferase; EC 2.6.1.13) probably has a major role in ornithine catabolism. It catalyzes the first step in the biosynthetic route to proline, the transfer of the ornithine α -amino group to α -ketoglutarate yielding glutamate and glutamate- γ -semialdehyde.

Rapidly growing hepatomas are characterized by marked decreases in the activities of ornithine carbamoyltransferase (45) and ornithine

aminotransferase (46) and an increase in the activity of ODC (47). The increased channeling of ornithine into putrescine and polyamines is specific for neoplastic cell growth and not associated with rapid cell proliferation *per se*. It should be emphasized, however, that despite the large increase in ODC activity and the decrease in ornithine carbamoyltransferase and ornithine aminotransferase, the latter activities were still 300-400-fold higher than that of ODC (45-47).

Carbamoylphosphate is a precursor of both arginine (via the urea cycle) and UMP (via the orotate pathway) (44, 48). A decrease in the ornithine carbamoyltransferase activity results in a decreased utilization of carbamoylphosphate for urea synthesis. Instead it can be used to an increasing extent for pyrimidine synthesis. In fact, a channeling of metabolites from the urea cycle to pyrimidine synthesis was found to parallel the increased rates of DNA synthesis and cell proliferation in a series of hepatomas (45).

2.4.2 Putrescine Synthesis (Fig. 3)

As mentioned above, decarboxylation of ornithine, a reaction catalyzed by ODC, is the only means of putrescine formation in mammalian cells. ODC is a pyridoxal 5'-phosphate-dependent enzyme (49). It does not require metal ions for activity, but is strongly dependent on low molecular weight thiols for catalytic activity (50, 51). In the absence of thiol compounds, the enzyme rapidly loses its activity, apparently as a result of polymerization (by formation of disulfide bridges) (49, 50). In studies of ODC, dithiothreitol is frequently added to stabilize the enzyme. It is not known, however, which physiological agents influence the red-ox status of ODC in the cell. Eukaryotic ODC is not susceptible to activation by any known low-molecular weight effectors (e.g. cyclic nucleotides).

With respect to substrate specificity, the rat liver ODC has been shown to strongly prefer a distance between the two nitrogen atoms of about 6Å, corresponding to a fully stretched conformation of ornithine (52). Nevertheless, ODC may decarboxylate lysine, yielding cadaverine (1,5-diaminopentane), as demonstrated in mouse kidney and rat liver (53, 54). The K_m for lysine was 100 times higher than that for ornithine and the maximum rate of cadaverine synthesis was about one quarter of that of putrescine synthesis (54). Under most physiological conditions, however, decarboxylation of lysine by ODC is negligible.

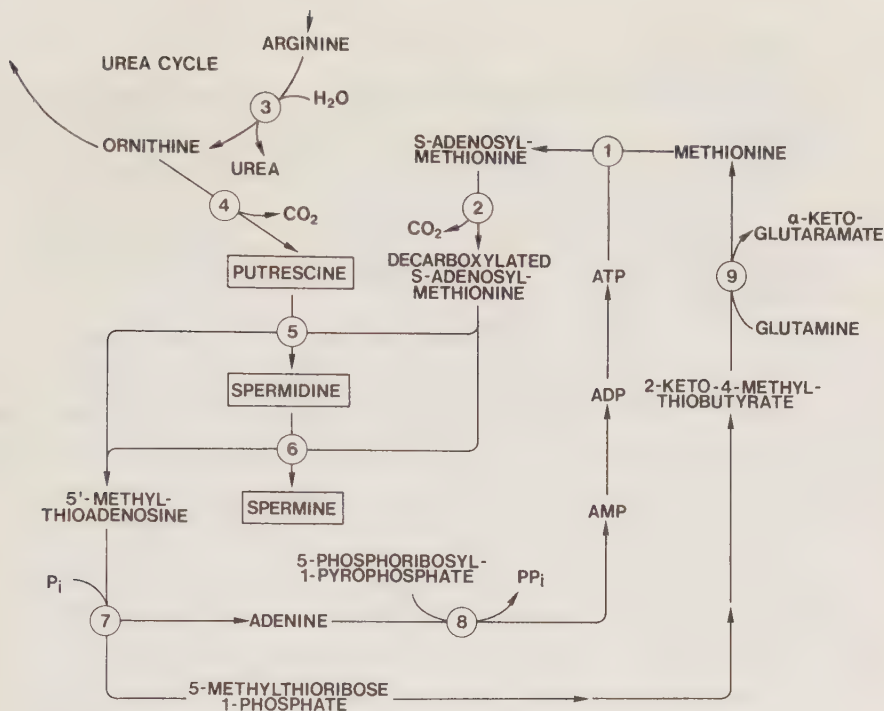


Figure 3. The polyamine biosynthetic pathway and its linkage to the salvage pathways of methionine and adenine nucleotide synthesis. 1) S-Adenosylmethionine synthase. 2) S-Adenosylmethionine decarboxylase. 3) Arginase. 4) Ornithine decarboxylase. 5) Spermidine synthase. 6) Spermine synthase. 7) 5'-Methylthioadenosine phosphorylase. 8) Adenine phosphoribosyltransferase. 9) Glutamine transaminase. The diagram shows how those parts of the S-adenosylmethionine molecule that are not used for polyamine production are effectively salvaged.

The ODC activity is very low in quiescent cells, but when cells are stimulated to grow it is rapidly induced (27, 55). However, even after stimulation, ODC constitutes but a small fraction of the total cellular protein, ranging from 0.0002% of the cytosolic protein in thioacetamide-stimulated rat liver (56) to 0.01% in androgen-stimulated mouse kidney (57).

ODC has been purified to homogeneity from rat liver (56) and mouse kidney (57, 58). The enzyme, which exhibited a molecular weight of 100-105000, appeared to be a dimer containing two probably identical 50-53000 molecular weight subunits with one active site on each subunit (56, 57). The K_m s for ornithine and pyridoxal 5'-phosphate were 75 and 0.3 μ M, respectively (57). The specific activities for the rat liver and mouse kidney enzymes were 1.1×10^6 and 3.0×10^6 nmol of CO₂/hr x mg

protein, respectively.

ODC appears to have the most rapid rate of synthesis and degradation among mammalian enzymes. Its half-life may be as short as 5-10 min (59, 60). The rapid change in activity, may be due to several control mechanisms; 1) transcriptional 2) translational and 3) post-translational, e.g. antizyme induction or release, and transition between active and inactive forms (61).

ODC is inhibited in a competitive fashion by its own product putrescine ($K_i \sim 1$ mM), and by spermidine ($K_i \sim 3$ mM) and spermine ($K_i \sim 9$ mM) (Fig. 4) (50, 62). These inhibition constants are higher than the steady state concentrations of the polyamines in most cells and tissues. Therefore, feedback inhibition of ODC by putrescine, spermidine and spermine is not likely to play a regulatory role *in vivo* (63). Putrescine, and to some lesser extent spermidine and spermine, may also induce the formation or release of a specific protein, ODC-antizyme which reversibly combines with and inactivates ODC (Fig. 4) (64-66).

It has been suggested that ODC is a multifunctional protein. Thus, ODC modified post-translationally by transglutamination and/or phosphorylation has been proposed to be involved in the regulation of RNA polymerase I activity (67, 68).

2.4.3 Spermidine and Spermine Synthesis (Fig. 3)

The aminopropyl moieties of spermidine and spermine are derived from methionine (49). The immediate aminopropyl group donor, however, is S-adenosyl-S-methyl-3-thioprop-1-ylamine, so-called "decarboxylated S-adenosylmethionine" (deSAM). The precursor of deSAM is S-adenosylmethionine (SAM), which is formed in a reaction catalyzed by S-adenosylmethionine synthase (ATP:L-methionine S-adenosyltransferase; EC 2.4.2.13). SAM is the methyl group donor for roughly a hundred trans-methylating enzymes. The decarboxylation of SAM, catalyzed by S-adenosylmethionine decarboxylase (SAMDC) (S-adenosyl-L-methionine carboxylase; EC 4.1.1.50), is considered to be the rate limiting step in the synthesis of spermidine and spermine. Once deSAM is formed, it is irreversibly committed to polyamine synthesis.

SAMDC contains a covalently linked pyruvate, which is essential for enzyme activity (69). Presumably, this pyruvoyl residue forms a Schiff's base with the substrate and thus facilitates the decarboxylation. The enzyme is independent of pyridoxal 5'-phosphate and divalent

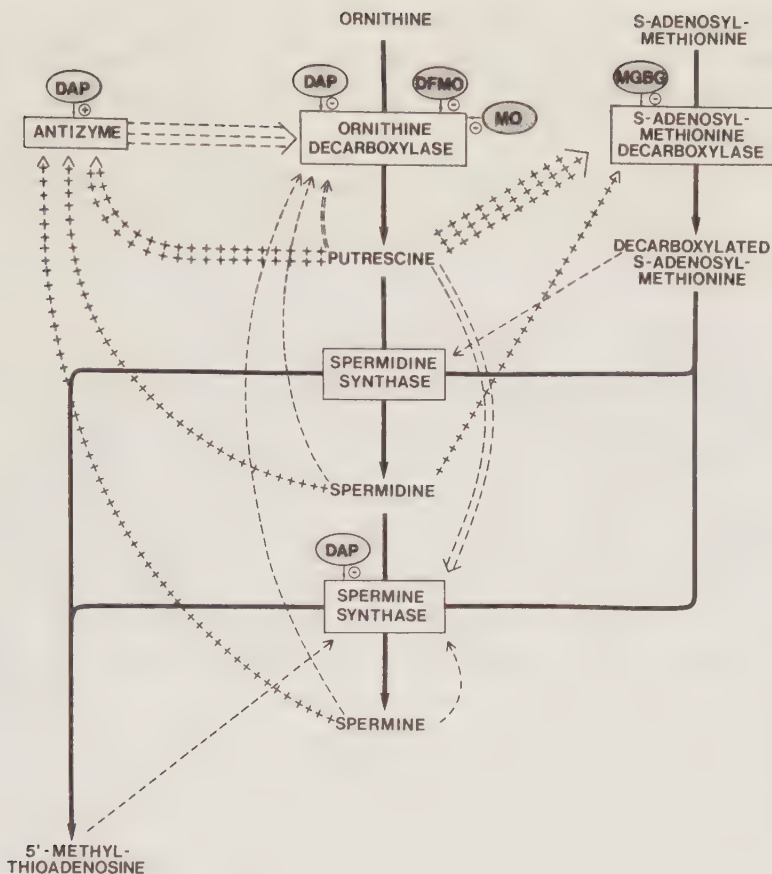


Figure 4. Positive (++++ and negative (----) regulation of the enzymes in the polyamine biosynthetic pathway. The width of the arrows roughly denote the relative extent of stimulation or inhibition. The sites of action of various polyamine synthesis inhibitors are indicated: DAP, 1,3-diaminopropane; DFMO, α -difluoromethylornithine; MO, α -methylornithine and MGBG, methylglyoxal-bis(guanylhydrazine).

metal ions for catalytic activity.

Upon stimulation of growth and differentiation the SAMDC activity increases precipitously (70). In unstimulated mammalian tissues it is present in very low amounts, ranging from 0.0007% of the soluble protein in liver to 0.015% in ventral prostate (55).

SAMDC has recently been purified to homogeneity from both rat liver and psoas muscle (71). The native enzymes, which exhibited a molecular weight of 68000, appeared to have two subunits with a molecular weight of about 32500. The specific activity was found to be

$3.8\text{--}3.9 \times 10^4$ nmol of $\text{CO}_2/\text{hr} \times \text{mg protein}$.

SAMDC is activated by putrescine (Fig. 4), which lowers the K_m for the substrate. For purified rat liver and psoas muscle SAMDC the K_a for putrescine was $25\text{--}30 \mu\text{M}$ and the K_m s for SAM (in the presence of a saturating putrescine concentration) were 50 and $100 \mu\text{M}$, respectively (71). Purified SAMDC was also activated by spermidine (apparently by the same mechanism as putrescine) but to a much lesser extent (Fig. 4) (71, 72).

Spermidine may also suppress SAMDC activity. Mamont et al. (73), proposed a post-translational site of action e.g. denaturation and/or degradation of the enzyme. Alhonen-Hongisto (74) on the other hand suggested that spermidine might regulate SAMDC at the transcriptional level. The activation of SAMDC in the cell is most likely accounted for by putrescine, while spermidine might act to suppress the enzyme.

Although the half life of SAMDC is longer than that of ODC, it is still much shorter than those of other enzymes (27, 75). The rapid changes in enzyme activity observed in growth-stimulated cells are due to variations in the amount of enzyme protein, rather than modulations in the catalytic activity of a constant amount of protein (76, 77).

Spermidine synthase (S-methyladenosylhomocysteamine:putrescine aminopropyltransferase; EC 2.5.1.16) catalyzes the transfer of an aminopropyl group from deSAM to putrescine, yielding spermidine (49). Subsequently, spermine synthase (S-methyladenosylhomocysteamine:spermidine aminopropyltransferase; EC 2.5.1.) catalyzes the transfer of an aminopropyl group from another molecule of deSAM to the primary amino group of the diaminobutane moiety of spermidine. Additional products in each of the two aminopropyl transferase reactions are 5'-methylthioadenosine (MTA) and one proton.

The activities of the aminopropyl transferases are usually much greater and the half lives are much longer than those of the decarboxylases. The activities of the aminopropyl transferases are regulated by the availability of the substrates, particularly by deSAM, even though the K_m s for this nucleoside are low (55). Spermidine synthase is subject to substrate inhibition at high concentrations of deSAM (K_i , 0.22 mM) (78). However, since the concentration of deSAM is normally extremely low (55, 79), spermidine synthase is not likely to be inhibited by this substrate in the cells.

Spermidine synthase has been purified to homogeneity from bovine

brain and rat ventral prostate (80). The native enzyme from rat ventral prostate, which exhibited a molecular weight of 73000, appeared to be composed of two probably identical 37000 molecular weight subunits. The K_m for deSAM was 1 μ M. K_m s for putrescine of about 30 and 100 μ M were obtained for spermidine synthase of the brain and prostate, respectively. Spermidine synthase was weakly inhibited by MTA, one of its products.

Spermine synthase, which is mainly found in eukaryotic cells, has been purified to homogeneity from bovine brain (81). The native enzyme, which exhibited a molecular weight of about 88000 was composed of two probably identical 45000 molecular weight subunits. The enzyme showed strict specificity for spermidine as the aminopropyl group acceptor. The K_m s for spermidine and deSAM were 60 and 0.6 μ M, respectively. The two major reaction products, spermine and MTA, inhibited the spermine synthase activity. MTA proved to be a powerful competitive inhibitor with respect to deSAM ($K_i \sim 0.3 \mu$ M) (Fig. 4). Putrescine inhibited the enzyme competitively with respect to spermidine ($K_i \sim 1.7$ mM) (Fig. 4). Purified spermine synthase had no requirements for metal ions or other cofactors (81).

2.4.4 Salvage Pathway of Adenine Nucleotide Synthesis (Fig. 3)

MTA is formed in amounts stoichiometric to the polyamine products in the spermine and spermidine synthase reactions. Nevertheless, the cellular concentrations of MTA are always very low (55, 79, 82). This is due to its rapid cleavage at the glycosidic bond by MTA phosphorylase (5'-methylthioadenosine:orthophosphate methylthioribosyltransferase), producing adenine and 5-methylthioribose 1-phosphate (55, 72, 82). A K_m value for MTA of 0.47 μ M was obtained for the purified rat liver enzyme (83). The adenine liberated in the MTA phosphorylase reaction can then react with 5'-phosphoribosyl-1-pyrophosphate to form 5'-AMP and pyrophosphate, in a reaction catalyzed by the widely distributed enzyme adenine phosphoribosyltransferase (5'-AMP:pyrophosphate phosphoribosyltransferase; EC 2.4.2.7) (81, 84). Thus, by means of this purine-salvage pathway, the adenine moiety of MTA can be incorporated into DNA or RNA or may re-enter the polyamine biosynthetic pathway (85, 86).

Although there are a number of other pathways for MTA synthesis, the spermidine and spermine synthase reactions represent the quantitatively most important ones (72, 85). This is interesting in view of the recent observation of Kamatani and Carson (87) that endogenously produced

adenine is derived nearly exclusively from the cleavage of MTA and hence from the polyamine biosynthetic pathway.

2.4.5 Salvage Pathway of Methionine Synthesis (Fig. 3)

The synthesis of methionine from MTA has been demonstrated in a variety of mammalian cell lines (88-90). The first step in this methionine salvage pathway is the formation of 5-methylthioribose 1-phosphate, catalyzed by MTA phosphorylase. 5-Methylthioribose 1-phosphate is then converted to the α -keto acid of methionine, which is in turn converted to methionine by a transamination reaction. Methionine is an essential amino acid for mammalian cells and the production of MTA could represent a significant loss unless it was possible to salvage this compound into the methionine pool.

2.5 POLYAMINE SYNTHESIS INHIBITORS

An obvious means of determining the physiological significance of the polyamines, and more specifically in which regulatory processes they may be involved, is to analyze the effects of specific depletion of the cellular polyamine content. With the recent development of inhibitors that interfere with the various steps in polyamine synthesis, it has become possible to specifically deplete cells of their polyamines. A large number of inhibitors have been synthesized, but only a few of the most specific ones are mentioned in this context. For a detailed survey of polyamine synthesis inhibitors the reader is directed to a review by Heby and Jänne (91).

2.5.1 Ornithine Decarboxylase Inhibitors

Because ornithine is a simple organic molecule, the possibilities for molecular manipulation are quite good. Accordingly, a number of ornithine analogs have been synthesized, some of which are efficient inhibitors of ODC (91).

α -Hydrazinoornithine (Fig. 5) was found to be a strong competitive inhibitor (with respect to ornithine), of mammalian ODC from various sources. The K_i value for rat ventral prostate ODC was 2 μ M (92). α -Hydrazinoornithine was found to inhibit other pyridoxal 5'-phosphate-dependent enzymes, but to a much lesser extent than ODC (49). In the presence of high concentrations of pyridoxal 5'-phosphate the extent of ODC inhibition was reduced (92).

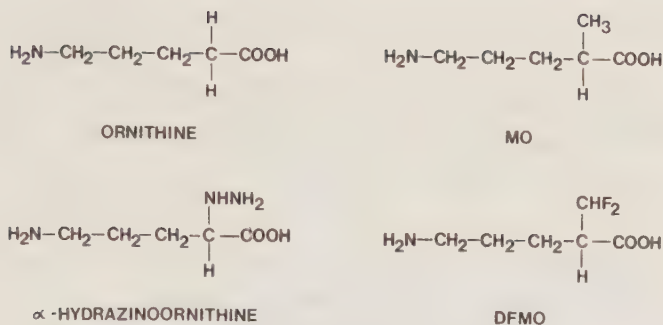


Figure 5. Molecular structures of three substrate analogs to ornithine. α -Hydrazinoornithine and α -methylornithine (MO) are reversible inhibitors and α -difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase.

α -Hydrazinoornithine has been used in a variety of systems and has been found to mainly reduce the putrescine content (91).

The synthesis of α -methylornithine (MO) was based on the fact that α -methyl amino acids are potent inhibitors of pyridoxal 5'-phosphate-dependent decarboxylases (93, 94). MO is a competitive inhibitor of mammalian ODC with a K_i value of about 20-40 μM for the racemic mixture (Figs. 4 and 5) (52, 93). The racemic mixture of MO has been used in most studies. The L-isomer, however, is a much more potent inhibitor than the D-isomer (52). The inhibition exerted by MO is not influenced by the ambient concentration of pyridoxal 5'-phosphate (93). MO has been used in a number of experimental systems to reduce the putrescine and spermidine content (91).

MO and α -hydrazinoornithine exert a number of unwanted effects, which result from secondary changes occurring in the polyamine biosynthetic pathway. These comprise 1) a stabilization of ODC, leading to a paradoxical accumulation of enzyme protein in the cell, 2) enhancement of SAMDC activity and 3) spermine accumulation (91).

α -Difluoromethylornithine (DFMO) (Figs. 4 and 5) (95) belongs to the novel class of enzyme-activated irreversible inhibitors known as k_{cat} inhibitors (96) or suicide enzyme inactivators (97). In Figure 6, the hypothetical mechanism of DFMO-mediated irreversible inactivation of ODC is shown in comparison to the normal ODC-mediated decarboxylation of ornithine, yielding putrescine (95).

DFMO specifically inactivates ODC and does not influence other

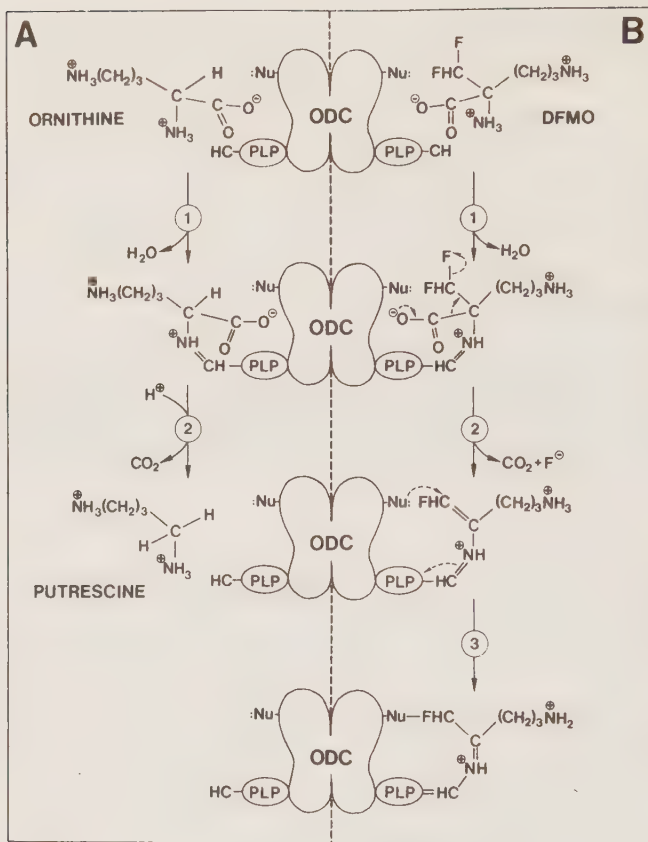


Figure 6. Hypothetical mechanism for α -difluoromethylornithine (DFMO)-mediated irreversible inactivation of ornithine decarboxylase (ODC) (B) shown in comparison to the normal decarboxylation of ornithine (A). First, a Schiff's base is formed between DFMO and pyridoxal 5'-phosphate (PLP) in the active site of the enzyme (B, Step 1). This reaction is reversible and basically the same as that occurring when the normal substrate is used (A, Step 1). Secondly, DFMO is enzymatically decarboxylated to yield a highly reactive electrophilic intermediate (a conjugated imine) (B, Step 2). During this step fluoride is eliminated. Thirdly, the electrophilic intermediate alkylates a nucleophilic residue (Nu), at or near the active site of ODC, thus covalently binding the inhibitor to the enzyme (B, Step 3).

enzymes employing ornithine as a substrate. The racemic mixture has been used in most studies, but only the L-isomer is active.

When DFMO-inactivated ODC was dialyzed against a buffer solution containing pyridoxal 5'-phosphate and dithiothreitol the enzyme activity was not regenerated. This demonstrates the irreversibility of the process. That the inhibitor is active site-directed is shown by the protec-

tive effects exerted by ornithine and putrescine against DFMO-induced inactivation of ODC (95).

DFMO has been used in cell cultures and intact animals to produce a rapid fall in the concentrations of putrescine and spermidine (91). The unwanted effects of DFMO comprise 1) enhancement of SAMDC activity and 2) spermine accumulation.

The concept of amine inhibition of ODC activity has been developed on the basis of the primary discovery of Pett and Ginsberg (98), that low concentrations of putrescine strongly inhibit ODC activity in a manner that does not involve direct product inhibition. In addition to putrescine, 1,3-diaminopropane (Fig. 7), a lower putrescine homolog, proved to be a very potent inhibitor of ODC (Fig. 4).

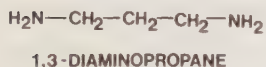


Figure 7. The molecular structure of the lower putrescine homolog, 1,3-diaminopropane.

1,3-Diaminopropane induces a very rapid decrease in ODC activity, almost comparable to that observed in response to cycloheximide. The rapidity of 1,3-diaminopropane-induced inactivation of ODC, suggests that it may act as a translational repressor of ODC (99). Some evidence suggests that it acts by preventing gene activation (99). It should be emphasized, however, that 1,3-diaminopropane does not exhibit any appreciable product inhibition of ODC *in vitro* (91).

1,3-Diaminopropane has been used in a number of systems to deplete the polyamine content (91).

One disadvantage of 1,3-diaminopropane is that it may fully or partly take over the intracellular functions of putrescine (91). Another is that it increases the SAMDC activity. It may also be a substrate of aminopropyl transferases.

2.5.2 S-Adenosylmethionine Decarboxylase Inhibitors

Methylglyoxal-bis(guanylhydrazone) (MGBG) (1-1'-((methylethane-diylidene)dinitrilo)diguandine) (Fig. 8) is an extensively studied polyamine synthesis inhibitor (100). The MGBG molecule consists of two planar aminoguanidine groups separated by an aliphatic skeleton. This compound was already in clinical use in cancer chemotherapy before its mechanism of action was known (100). First, the similarities between MGBG and spermidine were noted. They suggested the possibility that MGBG

might interfere with the biological functions of the polyamines (101). Subsequently, it was discovered that MGBG was an extremely potent inhibitor of eukaryotic SAMDC (Fig. 4) (102).

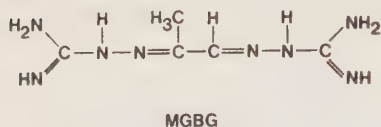


Figure 8. The molecular structure of methylglyoxal-bis(guanyldrazone) (MGBG), a potent S-adenosylmethionine decarboxylase inhibitor.

MGBG inhibits eukaryotic putrescine-activated SAMDC at micromolar concentrations, apparently by reversibly competing for the deSAM binding site (79, 91, 100). With respect to putrescine, the activator of SAMDC, MGBG behaves as an uncompetitive inhibitor (91, 100). In the absence of putrescine, MGBG is only a moderate inhibitor of SAMDC (72). MGBG effectively blocks the formation of spermidine and spermine in various cells grown in culture (91). In contrast, the cellular putrescine content is strikingly increased.

Several recent studies suggest that polyamine synthesis inhibition is not the only mechanism by which MGBG exerts its antiproliferative effects. Profoundly swollen mitochondria have been observed in cultured cells treated with micromolar concentrations of MGBG (100). MGBG treatment has also been shown to deplete cellular ATP pools and to reduce the adenylate energy charge (100). In addition, MGBG produces a number of unwanted effects, e.g. 1) stabilization of SAMDC, 2) stimulation of ODC, 3) inhibition of diamine oxidase and 4) inhibition of the polyamine uptake system (91, 100).

2.6 POLYAMINES AS REGULATORS OF CELL GROWTH

2.6.1 Polyamines in Proliferating Cells

Although polyamines have long been known to be present in cells, the elevation of their levels in proliferating mammalian cells was not demonstrated until 1965 by the studies of Dykstra and Herbst (103). They showed that partial hepatectomy resulted in marked increases in the putrescine and spermidine contents of the regenerating liver. Subsequent studies showed that one of the first changes to take place after partial hepatectomy was an increase in ODC activity (104).

It is now well known that growth stimulation of quiescent cells and tissues invariably results in an increased rate of polyamine synthesis (105). The enhanced synthesis and accumulation of polyamines upon

growth stimulation precedes or coincides with the initiation of DNA synthesis (104, 106).

The life cycle of a cell, which serves to double all the structural elements, involves two major processes: cell growth (interphase) and mitosis (M) (Fig. 9) (107, 108). The key event during cell growth is DNA synthesis. The observation that eukaryotic cells duplicate their DNA during a discrete interval, allows the interphase to be divided into three successive periods: G1, S (DNA synthesis) and G2. M is the fourth period of the cell cycle. After division, the daughter cells can either continue into the G1 phase of the next cell cycle or leave the cell cycle and enter a quiescent/resting (G0) phase. Cells in G0 are characterized by a continuous reduction in transcriptional activity as the cells progress deeper into this cell cycle phase (108). Cells in G0 may reenter G1, but it becomes increasingly difficult as they progress further into G0.

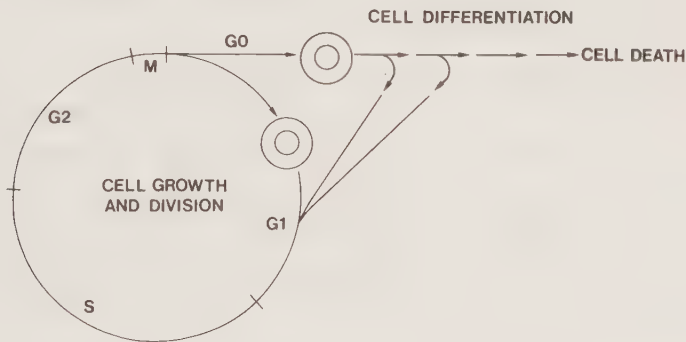


Figure 9. The cell cycle.

A number of investigators have studied the changes in the activities of ODC and SAMDC during the cell cycle (91, 104, 106). When cells are stimulated to grow from a quiescent state, a dramatic increase in ODC activity appears to be one of the first events to occur after entry into G1. The ODC activity reaches a first peak in early G1 and a second peak in late G1/early S, at the time of initiation of DNA synthesis (91, 106). A similar sequence of events has also been found for SAMDC (91). In addition to these two peaks of increased ODC activity, a third peak has been observed prior to cell division (91).

It should be mentioned that the early G1 peak of ODC activity has

not been observed in continuously proliferating cells and it has been suggested that this peak might be unrelated to cell proliferation (27). An apparently general phenomenon of cycling cells is the late G1/early S phase stimulation of ODC. The fact that there is a temporal correlation between this activity and the onset of DNA replication suggests that polyamines may play an important role in the regulation of DNA replication (91, 106).

Increased ODC and SAMDC activities are consistently followed by accumulation of putrescine and spermidine but not always of spermine (91).

2.6.2 Polyamine Synthesis Inhibition and Its Effects on DNA Replication

A large body of circumstantial evidence suggests that polyamine synthesis is associated with DNA replication and cell division. An obvious means of elucidating whether polyamines are absolutely required for these events is to study the consequences of specific polyamine depletion, which may be achieved by inhibition of the rate-controlling enzymes (ODC and SAMDC).

α -Hydrazinoornithine treatment of isoproterenol-stimulated mouse parotid glands (109), ethylphenylpropiolate-stimulated mouse skin (110), regenerating rat liver (111) and sarcoma-180 (112) was found to markedly reduce the increase in putrescine concentration without significantly affecting the spermidine and spermine contents. In these systems, DNA replication and cell proliferation were suppressed. Because putrescine formation and DNA replication were the only apparent events markedly reduced by α -hydrazinoornithine, it was suggested that putrescine accumulation is a prerequisite for DNA replication. The fact that putrescine administration reduced the inhibitory effects of α -hydrazinoornithine on DNA replication, supported the contention that putrescine was important for this process (109-112).

The antiproliferative effect of MGBG has been demonstrated in a variety of established cell lines, e.g. Chinese hamster ovary cells (113), L1210 cells (114), 9L rat brain tumor cells (115) and rat embryo fibroblasts (116). A dose dependent effect of MGBG on cell proliferation was demonstrated in L1210 cells (114). The polyamine profile produced by MGBG differs from that produced by ODC inhibitors. Thus, MGBG increases the cellular putrescine content and decreases the spermidine and spermine content (113-116). Addition of spermidine or spermine to MGBG-

-arrested rat embryo fibroblast cells resulted in a rapid resumption of proliferation (116).

MGBG treatment of phytohemagglutinin-stimulated human (117-119) and guineapig (120) lymphocytes and concanavalin A-stimulated bovine lymphocytes (121, 122) resulted in inhibition of DNA synthesis. The resulting polyamine profiles were similar to those of the MGBG-treated cells mentioned above. The dose-response curves for inhibition of DNA synthesis and of spermidine and spermine synthesis were parallel (117, 120). Inhibition of DNA synthesis was relieved by spermidine or spermine supplementation (117, 121). These experiments support the contention that the increased levels of spermidine generally seen in mammalian cells after growth stimulation are necessary for an optimal rate of DNA replication.

When treating cells with α -hydrazinoornithine or MGBG, conflicting results were obtained regarding the relative importance of putrescine, spermidine and spermine for DNA replication and cell proliferation. The discrepancies may be due to the fact that these compounds are not absolutely specific for polyamine synthesis (Sections 2.5.1 and 2.5.2). With the synthesis of MO and DFMO, however, it appeared as specific polyamine depletion was achievable.

MO treatment of rat hepatoma tissue culture cells (123), L1210 mouse leukemia cells (124) and human WI-38 fibroblasts (125) blocked the increases in putrescine and spermidine synthesis occurring following growth stimulation. The continuous depletion of cellular putrescine and spermidine accomplished by MO was followed by a reduction in the rate of cell proliferation. This anti-proliferative effect of MO was dose dependent (123).

Even though MO inactivates ODC specifically, it cannot be considered an ideal inhibitor of polyamine synthesis. One reason is that it increases the amount of ODC in the cell by stabilizing the enzyme. When MO is bound to ODC, the enzyme is probably less susceptible to proteolytic digestion, resulting in a marked accumulation of potential enzyme activity in the cell (126). MO may also increase the rate of ODC synthesis (126). Moreover, since ODC is merely reversibly inhibited by MO, it still possesses the capacity to decarboxylate ornithine. DFMO, on the other hand, which irreversibly inactivates ODC, should be more efficient than MO in inhibiting polyamine synthesis and therefore also in inhibiting polyamine-dependent growth-related events. Thus, in the

present study of the physiological role of the polyamines, DFMO was used to specifically deplete cells of their polyamines.

2.7 POLYAMINES AS REGULATORS OF CELL DIFFERENTIATION

Although the involvement of polyamines in mammalian cell proliferation is well documented, there have been relatively few studies on the possible role(s) of polyamines in differentiation. Differentiation can be defined as a phenotypic change of a cell, detected by biochemical and/or morphological parameters. The differentiated state depends on the transcription of genomic information and is a function of variable gene expression. There is considerable controversy concerning the mechanism(s) that initiate differentiation (127). Growth arrest in the G1 or G0 phase of the cell cycle appears to precede expression of the differentiated phenotype, which occurs in the G0 phase (Fig. 9). Differentiation is preceded by determination. Determination can be defined as a change in internal (genomic) character of a cell that commits it to a specialized course of differentiation. Very little is known about the events that commit unspecialized cells to differentiate into specialized cells.

The role of polyamines in differentiating mouse mammary gland in culture has been extensively studied by Oka and coworkers (128-130). During pregnancy, mammary epithelial cells undergo massive proliferation to form daughter cells that synthesize and secrete the milk proteins, casein and α -lactalbumin. The production of these proteins serves as a specific biochemical marker of mammary gland cell differentiation. When mammary gland explants from midpregnant mice were cultured with a combination of insulin, glucocorticoid and prolactin, significant increases in spermidine concentrations occurred prior to the accelerated synthesis of the milk proteins.

When the hormonally-induced stimulation of spermidine synthesis was inhibited by MGBG treatment, the synthesis of milk proteins was blocked. Upon the addition of spermidine to MGBG-treated mammary gland explants, however, the inhibition of milk protein synthesis was completely reversed. Neither putrescine nor spermine could reverse the inhibition. These results suggest that spermidine may be required for mouse mammary gland differentiation.

Rath and Reddi (131) have shown that the ODC activity and the polyamine levels increase during matrix-induced development and differentiation of cartilage, bone and bone marrow *in vivo*.

Friend erythroleukemia cells can be induced to differentiate along the erythroid pathway by a variety of compounds. Some of these inducers, including dimethylsulfoxide and hexamethylene bisacetamide, stimulate ODC activity (132). When this increase in polyamine synthesis was blocked by MGBG, MO or α -hydrazinoornithine, dimethyl sulfoxide- and hexamethylene bisacetamide-induced erythroleukemia cell differentiation was prevented. The inhibition of induced differentiation could be abrogated by putrescine, spermidine or spermine.

The aforementioned studies suggest that polyamines may act as positive effectors of cell differentiation, i.e. when polyamine synthesis was inhibited differentiation did not occur. In contrast, MGBG treatment of cultured keratinocytes was shown to induce differentiation (133). In this system polyamines appear to act as negative effectors of cell differentiation. The role of polyamines in cell differentiation appears to be complex, which accentuates the need to further study this process. In the present study, DFMO was used to evaluate the role of polyamines in the differentiation of a mouse embryonal carcinoma cell line, a model of mammalian embryogenesis.

2.8 POLYAMINE SYNTHESIS INHIBITORS AS ANTICANCER AGENTS

It is obvious that, polyamine synthesis inhibitors, because of their growth inhibitory effects, should be tested for cancer chemotherapeutic potential. Indeed, MGBG has been found effective in the treatment of acute myelocytic leukemia and lymphomatous diseases (134, 135).

Less obvious is the potential use of polyamine synthesis inhibitors as response modifiers of conventional cancer chemotherapeutic agents.

2.8.1 The Stabilizing Effects of Polyamines on DNA

Most of the knowledge about the molecular structure of DNA arises from X-ray diffraction studies of DNA fibers. Such studies have shown that the exact geometry of the DNA helix can vary depending on the nucleotide sequence (136). Thus, X-ray analysis of double-stranded DNA molecules, with a predetermined sequence of about 10 bases, showed that the helix adapts to A, B or Z conformation. Each helix conformation has its own intrinsic restrictions on DNA chain folding and structure.

The B conformation of DNA, the right-handed Watson and Crick double helix, is believed to be the predominant form in biological systems.

B DNA is partly characterized by a wide major groove and a narrow minor groove (136). The A conformation of DNA is also a right-handed helix, but it has a cavernous major groove and a very shallow minor groove (136). Much interest has been focused on the recent discovery that alternating GC sequences can form a very different left-handed double helix known as the Z DNA conformation (137, 138).

The protective effect of polyamines against heat denaturation of DNA was demonstrated in 1962 by Tabor (139). When polyamines were added to a calf thymus DNA solution, the DNA denaturation temperature increased markedly. Spermidine and spermine were far more active than putrescine in mediating the protective effect. The increase in denaturation temperature of DNA was considered to be the result of polyamine-DNA complex formation. It was suggested that the polyamines mediated the stabilizing effect by binding to the negatively charged phosphate groups of DNA.

Spermine has also been shown to stabilize lambda bacteriophage DNA against mechanical shearing (140) and to protect calf thymus DNA from enzymatic degradation (141). The addition of polyamines to native DNA, prevented N-methyl-N-nitrosourea-induced methylation of the N7 and O6 positions in guanine residues and of the N3 position in adenine residues (142).

Tsuboi (143) and Liquori (144) suggested a model for the binding of spermine to DNA. In their model, the primary and secondary amino groups of the trimethylene portion of spermidine and spermine are bound ionically to adjacent phosphate groups in one strand of DNA, while the tetramethylene portion stretches across the minor groove of the double helix to form a cross-bridge between phosphate groups in opposite strands (Fig. 10).

Subsequently, X-ray studies of spermine-DNA complexes showed that spermine indeed stabilized the naturally occurring B DNA conformation according to the Tsuboi-Liquori model (145). A complex between spermine and DNA in the A conformation has recently been demonstrated by X-ray analysis (146). In this complex, spermine must switch to another binding geometry than in B DNA.

The observed stabilization of DNA, suggests a specific interaction of polyamines with DNA, possibly as a result of cross bridges and charge neutralization. On the other hand, Bloomfield and Wilson (16) suggested that there is relatively little chemical specificity in the

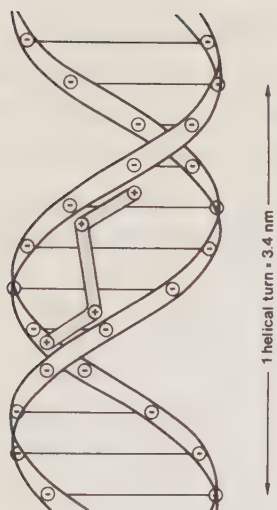


Figure 10. A schematic representation of the proposed binding of spermine in the minor groove of B DNA (the Tsuboi-Liquori model).

interaction of polyamines with DNA. According to the counter ion condensation theory, there would be a tight but delocalized binding of polyamines restricted to a quasi-cylindrical volume close to the DNA backbone, permitting the polyamines to diffuse along the length of the DNA molecule (16).

Although the B DNA helix appears to be the dominating form of DNA in the cell, evidence pointing to an important biological role for Z DNA is accumulating. It has been suggested that Z DNA is one of the elements regulating gene expression (147). Under physiological salt conditions, however, Z DNA is less stable than B DNA (147). Interestingly, the Z DNA conformation is effectively stabilized by spermine and to a lesser extent by spermidine (147). Methylation of cytosine in the C5 position also results in a marked stabilization of Z DNA (147). When DNA has been methylated, very low concentrations of spermidine and spermine induce the transition between B and Z DNA (148). The preferential binding of spermine to GC-rich regions of DNA suggests an involvement of polyamines in B to Z transition of the DNA helix (149).

The stabilizing effects of polyamines on A, B and Z DNA are well documented. This raises the question whether cellular polyamine depletion causes destabilization of DNA. If so, it may be possible to alter the effects of various agents that exert their cytotoxicity through interaction with DNA. In the present study, DFMO was used to deplete cells of their polyamines, before treatment with various DNA damaging agents.

2.8.2 Cell Cycle Specific Block by Polyamine Synthesis Inhibition

As mentioned earlier (Section 2.6.1), polyamine synthesis changes according to a certain pattern during the cell cycle. The increase in ODC activity during late G1/early S indicates that polyamines may play an important role in the progression of cells into the S phase.

When polyamine synthesis was inhibited by MGBG treatment, normal cells (WI-38, rat embryo, mouse 3T3 and human skin fibroblasts) were blocked in the G1 phase of the cell cycle (113, 116, 150). In contrast, MGBG treatment of transformed cells (HeLa, SV-3T3 and CHO cells) resulted in S phase block.

The conclusion drawn from these studies was that normal diploid cells recognize the MGBG-induced polyamine limitation and stop in G1. The transformed cells, on the other hand, do not recognize the MGBG-induced polyamine limitation and continue to progress through the cell cycle, but a majority fail to complete DNA synthesis and are thus arrested in S. Even though there may be exceptions to this rule (115), it was proposed that the differential effect of polyamine synthesis inhibition on the cell cycle traverse of normal and transformed cells could be exploited to preferentially kill transformed cells with an S phase specific drug. This suggestion was evaluated in the present study, by exposing a tumor cell line to DFMO before treatment with an S phase specific drug.

3. THE PRESENT STUDY

3.1 SPECIFIC AIMS

Many inhibitors of polyamine synthesis exert effects on reactions unrelated to polyamine metabolism and/or they induce secondary changes in the polyamine biosynthetic pathway. In the present study, DFMO, an enzyme-activated irreversible inhibitor of ODC, was used to more specifically deplete cells of polyamines in order to study their role in cell growth (I).

To determine whether polyamine deficiency is indeed the cause of growth inhibition observed in DFMO-treated cells, putrescine and other divalent cations were added to the culture medium, and their capacity to reverse the antiproliferative effect was assessed (II).

Endogenous adenine is nearly exclusively derived from the spermidine and spermine synthase reactions. Because these aminopropyl transferase reactions do not take place to any appreciable extent in DFMO-treated cells, it seemed likely that the growth inhibition observed might be partly caused by perturbations in the adenine nucleotide pools. Therefore, the cellular ribonucleotide and deoxyribonucleotide contents were analyzed in DFMO-treated cells (III).

To determine whether DFMO treatment affects cellular morphology (mitochondrial structure in particular), as does MGBG, transmission electron microscope studies were performed. In addition, because cellular ATP pools are reduced in MGBG-treated cells, the mitochondrial function was evaluated during DFMO treatment (IV).

The role of polyamines in cell differentiation is complex and not well understood. The present study addresses the question whether polyamine depletion of mouse embryonal carcinoma cells causes growth inhibition and as a consequence induces or prevents their differentiation (V).

Because the polyamines appear to exert an important stabilizing effect on DNA, polyamine depletion may alter the DNA structure, possibly modifying the response of cancer chemotherapeutic agents that exert their cytotoxicity by damaging DNA. To investigate this possibility, polyamine-depleted cells were exposed to cancer chemotherapeutic agents that interact with DNA by various mechanisms (VI, VII).

On the basis of some experimental evidence, it has been hypothesized that polyamine depletion blocks normal cells in G1 and transformed cells in S. Such a differential effect may be exploited to preferentially kill transformed cells with an S phase specific cytotoxic agent. To investigate this possibility, the cytotoxicity of an S phase specific drug was evaluated in polyamine-depleted tumor cells (VIII).

3.2 POLYAMINES AS REGULATORS OF CELL GROWTH - EXPERIMENTAL SYSTEMS

By specifically depleting cells of polyamines it should be possible to study their role in cell growth. Thus, Ehrlich ascites tumor cells (Section 3.2.1) were inhibited in their ODC activity (Section 3.5.3) by DFMO treatment, and the effects on polyamine content (Section 3.5.2) and cell proliferation (Section 3.5.1) were analyzed. The effect of polyamine depletion on the progression of cells through the cell cycle was studied by flow cytometry (Sections 3.5.4 and 3.5.5).

Since the polyamine biosynthetic pathway is interconnected with other biological pathways (e.g. *de novo* synthesis of adenine and all methylation reactions that use SAM as substrate), these may also be affected by inhibition of polyamine synthesis. To determine to what extent other metabolites are affected, and thus possibly involved in DFMO-mediated growth inhibition, the levels of SAM (Section 3.5.6), deSAM (Section 3.5.6), ribonucleotides (Section 3.5.7) and deoxyribonucleotides (Section 3.5.8) were determined.

Finally, to determine whether DFMO treatment affects cellular morphology (mitochondrial structure in particular), as does MGBG, transmission electron microscope studies (Section 3.5.9) were performed on Ehrlich ascites tumor cells and rat brain tumor cells (Section 3.2.2). In addition, mitochondrial function was evaluated by pyruvate utilization analysis (Section 3.5.10).

3.2.1 Ehrlich Ascites Tumor Cells (I-III)

The Ehrlich ascites tumor was originally derived from a solid transplantable mouse mammary carcinoma (151). It was successfully converted to an ascites tumor, implying that the tumor cells grew in suspension in the peritoneal cavity (152). The tumor was distributed to laboratories all over the world by Dr. Hans Lettré. For the present studies, a hyperdiploid subline, Ehrlich-Lettré-Diploid (ELD) Copenhagen, has been used. This strain of the tumor was obtained from the Department of Genetics at the University of Lund (153). Since then the tumor has been kept in inbred NMRI male mice by weekly intraperitoneal transplantations.

To establish cell cultures, tumor cells were withdrawn from the peritoneal cavity of a mouse harboring a 6-day tumor and were adapted to suspension culture growth (without agitation). The cultures were incubated at 37°C in a water-saturated atmosphere of 5% CO₂ in air. Swim's

S-77 growth medium, containing 10% fetal calf serum and the various supplements described in paper I, was used. ELD cells were grown in this medium for about 30 passages without any apparent changes in growth characteristics (I, II).

The ELD cells were found to grow equally well in the presence of 0.2% bovine serum albumin as in the presence of 10% fetal calf serum. Therefore, ELD cells are now maintained in a mixture of Ham's F12 medium and Eagle's minimum essential medium (1:1 by volume) supplemented with 0.2% bovine serum albumin, penicillin (50 IU/ml)-streptomycin (50 µg/ml) mixture and 20 mM NaHCO₃. ELD cells have been grown in this medium for more than 200 passages (III).

3.2.2 Rat Brain Tumor Cells (IV)

The 9L rat brain tumor, which may be grown *in vivo* and *in vitro*, has been developed into a standard model for brain tumor research. It was originally derived from an N-methyl-N-nitrosourea-induced rat brain tumor. The primary tumor was grown in culture and then in the flanks of newborn rats. After another growth cycle *in vitro* and *in vivo* a frozen tumor bank was created. The 9L tumor was originally diagnosed as an astrocytoma, but is presently described as either a gliosarcoma or a sarcoma.

Since the characteristics of the 9L cells appear to change when they grow *in vitro* for more than 6 months, a new culture was initiated every 3 months from the frozen tumor bank. The cells were grown in monolayer culture in Eagle's minimum essential medium supplemented with 1 mM non-essential amino acids, gentamicin (50 µg/ml) and 10% newborn calf serum. All incubations were carried out at 37°C in a water-saturated atmosphere of 5% CO₂ in air.

3.3 POLYAMINES AS REGULATORS OF CELL DIFFERENTIATION - EXPERIMENTAL SYSTEM

To determine whether the polyamines play a role in embryonal carcinoma cell differentiation, a model system of mammalian embryogenesis (Section 3.3.1), the cells were inhibited in their ODC activity by DFMO treatment. The effects on polyamine content (Section 3.5.2), cell proliferation (Section 3.5.1) and cell cycle kinetics (Sections 3.5.4 and 3.5.5) were analyzed. Morphological criteria and plasminogen activator activity (Section 3.5.11) were analyzed to assess differentiation

of DFM0-treated embryonal carcinoma cells.

3.3.1 Embryonal Carcinoma Cells (V)

Teratomas are tumors containing chaotic associations of differentiated tissues (154, 155). These tissues, which may include derivatives of all three germ layers, are formed by differentiation of a pluripotent stem cell called embryonal carcinoma cell. The embryonal carcinoma cell is responsible for the malignancy of the tumor (156), but the differentiated tissues to which it gives rise are nonmalignant.

The F9 embryonal carcinoma cell line was isolated from embryoid bodies of the transplantable tumor OTT 6050-970, a pluripotent teratoma, which originated from the grafting of a 6-day male mouse embryo to the testis of a strain 129 mouse (157). F9 cells have been designated "nullipotent" because they do not form the differentiated tissues diagnostic of teratocarcinomas, when propagated in the mouse as a solid subcutaneous tumor. However, Strickland and Mahdavi (158) recently showed that retinoic acid treatment induces F9 cells to differentiate into parietal endoderm. The differentiated cells are characterized by morphological alterations, induction of plasminogen activator production, sensitivity to dibutyryl cAMP and increased synthesis of collagen-like proteins (158).

The F9 embryonal carcinoma cells used were kindly provided by Professor Nils R. Ringertz (Karolinska Institutet, Stockholm). The F9 cells were grown in monolayer culture in Dulbecco's Modified Eagle Medium containing 20% fetal calf serum and the various supplements described in paper V. The cultures were incubated at 37°C in a water-saturated atmosphere of 5% CO₂ in air. In order to retain the undifferentiated malignant state characterized for embryonal carcinoma cells, F9 cells were subcultured every second day and plated in gelatinized petri dishes.

3.4 POLYAMINE SYNTHESIS INHIBITORS AS ANTICANCER AGENTS - EXPERIMENTAL SYSTEM

3.4.1 Rat Brain Tumor Cells (VI-VIII)

The stabilizing effects of polyamines on DNA are well-documented. Consequently, polyamine depletion should result in DNA destabilization, and may therefore alter the cytotoxicity of agents that exert their effects on DNA. The effects of polyamine depletion on the cytotoxicity caused by various DNA damaging agents was evaluated using 9L cells.

The cultivation of 9L cells is described above (Section 3.2.2). Cytotoxicity was evaluated by a colony forming efficiency assay (Section 3.5.12).

3.5 PREPARATIVE AND ANALYTICAL PROCEDURES

3.5.1 Collection and Counting of Cells (I-III, V-VIII)

Cells were counted in a Coulter counter (I, II), in a hemocytometer (III, V) or in a Royco cell counter (VI-VIII). ELD cells were counted after removal of an aliquot of the cell suspension. 9L cells and F9 cells were counted after the monolayers had been dissociated with trypsin into a monodisperse cell suspension. Cells intended for biochemical analyses were harvested by centrifugation and the cell pellets were stored at -20°C , except those intended for ODC assay, which were stored at -70°C . Extracts for SAM, deSAM, ribonucleotide and deoxyribonucleotide analysis were prepared immediately after pelleting the cells and were stored at -70°C .

3.5.2 Polyamine Analysis (I-III, V, VI)

Quantitative polyamine analysis were performed in cell homogenates obtained by sonication in 8% 5-sulfosalicylic acid (VI), in 0.2M perchloric acid (PCA) (II, V) or in 10 mM Tris-HCl buffer (pH 7.2) containing 5 mM dithiothreitol, 0.5 mM Na_2EDTA and 0.05 mM pyridoxal 5'-phosphate (I). An aliquot of the latter was mixed with an equal volume of 0.4M PCA. Yet another procedure was followed in III, where the cells were sonicated in 25 mM H_2SO_4 and PCA added to a final concentration of 0.2M. All acid extracts were kept on ice for 1 hr and were then centrifuged at 1000xg for 10 min.

A variety of techniques are presently available for the quantitative analysis of the polyamines (159, 160). Thin-layer chromatography (I, II, V) (161), ion-exchange chromatography using an amino acid analyzer (VI) (162), and high-performance liquid chromatography (III) (163) were used in the present study.

Only the thin-layer chromatographic method, which was used in most analyses, is described (I, II, V). Polyamines were allowed to react with an excess of 1-dimethylamino-naphthalene-5-sulfonyl-chloride (DANS-Cl) according to the method of Seiler and Wiechman (161). Thus, a 200- μl -aliquot of each PCA extract was mixed with 400 μl of a DANS-Cl solution (30 mg/ml in acetone) and 100 μl of a saturated Na_2CO_3 solution.

Na_2CO_3 was added since DANS-Cl reacts more favorably with amino groups during alkaline conditions (around pH 10). DANS-Cl reacts rapidly with primary and secondary amino groups under suitable conditions. However, to ascertain quantitative dansylation of the polyamines, the reaction mixtures were kept for 16 hr in the dark at room temperature. Since DANS-Cl is easily hydrolyzed by silica gel to DANS-OH (161), which causes blue-green fluorescent streaks on the chromatogram, 100 μl of a proline solution (150 mg/ml) was added to remove excess DANS-Cl. After 30 min in the dark, the dansylated polyamines were extracted into 500 μl of toluene by vigorous shaking. A 5- μl -aliquot of each toluene extract was applied to an activated (1 hr at 110°C) Silica Gel 60 thin-layer chromatographic plate with a Hamilton microsyringe pipet. Mixtures of the three polyamines, dansylated in parallel with the samples and applied to each plate, were used as standards.

The thin-layer chromatographic plates were developed in ethyl-acetate/cyclohexane (2/3, v/v) (I, V) in the dark for about 90 min and were then dried for 30 min in a desiccator in the dark. Since pyridoxal 5'-phosphate interferes with the polyamines in the aforementioned elution medium, chloroform/isopropanol (25/1, v/v) had to be used whenever pyridoxal 5'-phosphate was present in the homogenization medium (I).

The dansylated polyamines were analyzed using an Aminco-Bowman spectrophotofluorometer equipped with a Thin-Film Chromatography Scanner and an X-Y-recorder. The excitation maximum was 340 nm and the emission maximum 505 nm for the dansylated polyamine derivatives.

3.5.3 Ornithine Decarboxylase Assay (I, II)

For this assay, the cells were disrupted by sonication in 10 mM Tris-HCl buffer (pH 7.2) containing 5 mM dithiothreitol, 0.5 mM Na_2EDTA and 0.05 mM pyridoxal 5'-phosphate. The ODC activity was measured by trapping $^{14}\text{CO}_2$ released enzymatically from DL-(1- ^{14}C)-ornithine (50).

The reaction mixture (1 ml) contained 10 mM Tris-HCl (pH 7.2), 5.5 mM dithiothreitol, 0.2 mM pyridoxal 5'-phosphate, 0.05 mM Na_2EDTA and 1 mM L-ornithine (saturating concentration). To start the reaction, 0.5 μCi of DL-(1- ^{14}C)ornithine monohydrochloride (specific activity 59 mCi/mmole) was added. The reaction was carried out in plastic test tubes equipped with a rubber stopper. The $^{14}\text{CO}_2$ released during the incubation at 37°C was trapped in 100 μl of 1M Hyamine hydroxide, contained in a polypropylene well attached to the rubber stopper. By the injection of

1 ml of 40% trichloroacetic acid through the rubber stopper, the reaction was halted and bound $^{14}\text{CO}_2$ was released from the reaction mixture. A further incubation for 30 min at 37°C was allowed to ensure that all $^{14}\text{CO}_2$ released during the enzymic reaction, was trapped in the Hyamine hydroxide solution. The well and its content was then removed and placed in a plastic counting vial containing 10 ml of liquid scintillation fluid. Radioactivity was assayed with a liquid-scintillation spectrometer. Corrections were applied for radioactivity of non-enzymic control samples (100 μl of the homogenization medium replacing the cell extracts) in all experiments.

3.5.4 Cell Preparative Procedures for Flow Cytometric Analysis (III, V, VIII)

Quantitative fluorescent staining and flow cytometric analysis of cellular DNA have provided a rapid and reliable approach for assessing relative DNA contents of cells, in order to determine their cell cycle position. A number of different DNA staining techniques are currently being used in conjunction with flow cytometry (164). Two fluorochromes, propidium iodide and chromomycin A3, possessing different mechanisms of binding to DNA have been used in this study. Before staining with either of the fluorochromes, single-cell suspensions ($1\text{--}2 \times 10^6$ cells) were fixed in 1 ml of absolute ethanol (-20°C) and stored at 4°C until analysis.

Propidium iodide, an analog of ethidium bromide, selectively and quantitatively intercalates into the double-stranded regions of DNA and RNA (165). It can be used as a quantitative stain for cellular DNA after treatment of ethanol-fixed cells with RNase (166). The following staining procedure was used in two studies (III, V). After pelleting, the cells were resuspended in 1 ml of 0.1% RNase (75 U/ml) in Buffer A (0.1M Tris-HCl buffer containing 70 mM NaCl and 5 mM Na_2EDTA , adjusted to pH 7.5 and filtered through a $0.45\ \mu\text{m}$ Millipore filter) and kept at room temperature for 5 min. After pelleting, the cells were resuspended in 1 ml of 0.5% pepsin (5 E/ml) in 55 mM HCl and incubated for 10 min at 37°C . Pepsin treatment substantially increases dye binding to DNA and it also eliminates cytoplasmic fluorescence (167). The resulting cell nuclei were cooled on ice (5 min), pelleted and resuspended in 1 ml of Buffer A and passed through a nylon mesh with a pore size of $50\ \mu\text{m}$, using a 1 ml syringe. For quantitative fluorescent staining, pelleted cells were finally resuspended in 1 ml of 0.005% (75 μM) propidium

iodide in Buffer A supplemented with 0.6% Nonidet P40. NaCl was kept at concentrations higher than 10 mM, to eliminate binding of the fluorochrome to residual RNA and secondary sites in DNA. The nonionic detergent (Nonidet P40) was added to the staining solution to remove residual cytoplasm, that might cause clumping and nonspecific staining (168). The stained cell nuclei were finally passed through a 50 μm nylon mesh. Between each step in the protocol described, the cells were centrifuged at 250xg for 5 min and the supernatant decanted. Unless specifically stated, all procedures were performed at 0-4°C.

Chromomycin A3 is an antibiotic which specifically binds to double helical DNA, but not to ribonucleic acids (164). In contrast to propidium iodide, chromomycin A3 does not intercalate into DNA. Although highly stable chromomycin A3-DNA complexes are formed, the binding appears noncovalent in nature. Chromomycin A3 has been shown to preferentially bind to GC rich regions of DNA. The following staining procedure was used (VIII). Ethanol-fixed cells were pelleted and resuspended in staining solution (20 $\mu\text{g}/\text{ml}$ of chromomycin A3 in 15 mM MgCl_2) (169). After 20 min of staining, the cells were pelleted and resuspended in distilled water to give a final concentration of 2×10^6 cells per ml.

3.5.5 Cell Kinetic Analysis by Flow Cytometry (III, V, VIII)

Propidium iodide-stained nuclei, at a density of about 1×10^6 per ml, were analyzed on a Cytofluorograph System 50-H (Ortho Instruments) at the Department of Oncology, University of Lund (III, V). This flow cytometer is equipped with a 4 W argon-ion laser (Model 95). The laser line at 488 nm (450 mW output) was used for excitation. Fluorescence emission was detected between 625 nm and infrared. About 2×10^4 nuclei from each preparation were analyzed. The impulses were sorted by a 512 multichannel analyzer and processed with a PDP 11/34 computer. Nuclear doublets were excluded by electronic threshold settings.

In the DNA histograms obtained, the peak at approximately twice the modal channel number of G1 cell nuclei represents G2 cell nuclei. Obviously, mitotic cell nuclei should appear in this second peak. However, since most mitotic cells lack a nuclear membrane, they are likely to be destroyed by pepsin (167). The nuclei of late-telophase cells may separate and appear in the first peak of the histogram. The percentages of cells in each phase of the cell cycle were derived from the DNA distributions essentially as described by Dean (170).

3.5.6 S-Adenosylmethionine and Decarboxylated S-Adenosylmethionine Analysis (III)

The ELD cell content of SAM and deSAM was determined by a high-performance reversed-phase liquid chromatography method described by Wagner et al. (171). A Waters Associates high-performance liquid chromatography system was used to separate SAM and deSAM at 40°C on a μ Bondapak C₁₈ reversed-phase column according to the following procedure. A linear gradient was used, starting with initial conditions consisting of 85% Buffer A (0.1M NaH₂PO₄, 8 mM octane sulfonate, 0.1 mM EDTA and 2% acetonitrile, pH 2.55) and 15% of Buffer B (0.14M NaH₂PO₄, 8 mM octane sulfonate and 30% acetonitrile, pH 3.10). The final conditions, reached after 30 min, consisted of 15% A and 85% B. SAM and deSAM were detected by their absorbance at 254 nm and were quantitated by computer integration of the area under each chromatographic peak. Octane sulfonate was used as the ion-pairing agent.

3.5.7 Ribonucleotide Analysis (III)

The ELD cell ribonucleotide levels were analyzed by high-performance liquid chromatography. The method used is described in detail in paper III and will only be briefly summarized. PCA extracts of ELD cells were neutralized with KHCO₃ and KOH. The ribonucleotides were separated at room temperature on a polyanionic column with a linear gradient of 10-800 mM (NH₄)₂HPO₄/NH₄H₂PO₄-buffer, pH 7.0, using a Varian high-performance liquid chromatography system. The ribonucleotides were detected by their absorbance at 254 nm and were identified by the retention times obtained for the appropriate standards, injected alone or mixed with the samples. The ribonucleotides were quantitated by computer integration (and by cutting and weighing) of the area under each chromatographic peak.

3.5.8 Deoxyribonucleotide Analysis

An aliquot of 0.3M ice-cold PCA was added to the pelleted ELD cells, which were disrupted by repeated pipetting for 5 min at 0°C. Precipitated protein was removed by centrifugation at 1000xg for 10 min at 4°C. The supernatants were supplemented with 1.5 volumes of 0.5M tri-N-octylamine in 1,1,2-trichlorotrifluoroethane. After vortexing several times during a 5 min period at 4°C, the samples were centrifuged at 1000xg for 3 min. The upper aqueous layer, which contained

the acid-soluble nucleotides, was carefully removed and stored at -70°C . The deoxyribonucleotides were then quantitatively analyzed by an isotope method (172, 173).

3.5.9 Cell Preparative Procedures for Electron Microscopy (IV)

The preparation of 9L cells for electron microscopy is described in detail in paper IV and will not be mentioned in this context.

ELD cells, intended for electron microscopy, were harvested by centrifugation at $500\times g$ for 5 min at room temperature. The cells were fixed in 2.5% glutaraldehyde in 0.2M cacodylate buffer (pH 7.3) for 1 hr at 4°C . The glutaraldehyde-fixed cells were post-fixed in a solution of 1% osmium tetroxide in 0.2M cacodylate buffer (pH 7.3) for 1 hr at 4°C . Following rinsing in cacodylate buffer, the cells were dehydrated and stained in ethanol (90%) containing 1% phosphotungstic acid and 0.5% uranyl acetate. The cells were embedded in Vestopal W and sectioned for electron microscopy. The cells were examined in a Zeiss EM10 electron microscope at the Unit of Electron Microscopy, Department of Zoology, University of Lund.

3.5.10 Pyruvate Utilization Analysis (IV)

Pyruvate utilization, a measure of mitochondrial function, was quantitated by determining the amount of $^{14}\text{C}\text{O}_2$ released from cells incubated in the presence of $(2\text{-}^{14}\text{C})$ pyruvate (174). Active pyruvate dehydrogenase and a functioning citric acid cycle are required to convert pyruvate to acetyl-CoA and then to CO_2 . To release the label in position 2 requires that the pyruvate cycles twice through the mitochondrial tricarboxylic acid cycle. Because the radioactive carbon is in position 2 of the pyruvate molecule, pyruvate carboxylase does not interfere with the assay.

Cells were trypsinized, washed once in phosphate-buffered saline (PBS) and resuspended in PBS to a density of 10^7 cells/ml. $(2\text{-}^{14}\text{C})$ Pyruvate (7.7 mCi/mmol, New England Nuclear, Boston, MA) was diluted to 0.5 $\mu\text{Ci/ml}$ in PBS. Twenty μl of the diluted radioactive pyruvate solution was added to 80 μl of the cell suspension in a test tube, which was immediately fitted with a rubber stopper. The $^{14}\text{C}\text{O}_2$ released during a 30-min incubation period at 37°C (with agitation) was trapped in 100 μl of 0.1M NaOH contained in a polypropylene well attached to the rubber stopper. To stop the reaction, 0.5 ml of 20% sulfuric acid was injected

through the rubber stopper. The tubes were then incubated for 1 hr, to ensure complete capture of $^{14}\text{CO}_2$ released. The wells and their contents were removed and placed in plastic counting vials containing 10 ml of liquid scintillation fluid. Radioactivity was assayed with a liquid-scintillation spectrometer. Corrections were applied for radioactivity of non-enzymic control samples (100 μl of PBS replacing the cell suspension) in all experiments.

3.5.11 Plasminogen Activator Assay (V)

Plasminogen activator is a serine protease, which converts the plasma zymogen plasminogen to the active protease plasmin. Plasmin was initially found to be essential for the lysis of fibrin, but its specificity is much like that of trypsin and it is able to degrade other proteins as well. Plasminogen activator is produced by a wide variety of normal and transformed cells (175). Since plasmin has a broad substrate specificity and plasminogen is present in plasma and interstitial fluids, plasminogen activator has been implicated in many physiological and pathological processes, e.g. tissue remodeling, cell migration, inflammatory tissue damage and tumor cell invasion (176).

As mentioned earlier, plasminogen activator production is a marker of F9 embryonal carcinoma cell differentiation into parietal endoderm. Thus, the plasminogen activator assay was used to study the effect of DFM0 treatment on F9 embryonal carcinoma cell differentiation (V).

A radial caseinolysis assay in agarose was used to determine plasminogen activator activity (177). Cell homogenates were applied into wells, cut in opaque casein- and plasminogen-containing agarose gels. Sodium dodecyl sulphate was added to the homogenates in order to inactivate protease-inhibiting substances that may be present. Sodium dodecyl sulphate denatures protease inhibitors irreversibly, while its inhibitory effects on plasminogen activator and plasmin are reversible and dilute out during the radial diffusion of the samples. The agarose gels were incubated at 37°C for 3 days. The diameters of the clear circular areas, resulting from proteolysis, were proportional to the amount of plasminogen activator activity in the sample. Human urokinase, a plasminogen activator purified from urine, was used as standard.

3.5.12 Colony Forming Efficiency Assay (VI-VIII)

A cell is considered clonogenic if it can proliferate to form a colony. Clonogenicity (colony forming efficiency) is determined *in vitro* by 1) plating monodisperse cells at a known, low density, 2) allowing growth for several cell generations to obtain clonal colony formation and 3) calculating the number of colonies exceeding a predetermined size in percent of the number of cells inoculated (178).

The following colony forming efficiency assay was used to assess the cytotoxicity caused by DNA damaging agents, used alone or in combination with DFM0. After drug treatment, 9L cells were trypsinized (0.25% trypsin and 0.02% Na₂EDTA in Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution) and counted. One-ml-aliquots, containing a known number of cells, were plated in 4 ml of medium into 50 mm petri dishes, on a monolayer of 5x10⁴ irradiated 9L feeder cells. For each drug dose used, cells were plated at 3 different densities (4 petri dishes each). The petri dishes were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 14 days. All colonies were simultaneously fixed and stained (5 min) by the addition of approximately 2 ml of 0.2% crystal violet in absolute ethanol. The petri dishes were then rinsed with water to remove excess stain. A colony was defined as a cluster of 50 cells or more.

To evaluate cytotoxic effects of various drugs, plating efficiencies, surviving fractions, dose enhancement ratios (a measure of increased cell kill) and dose modification ratios (a measure of decreased cell kill) were calculated according to the following formulas:

$$\text{Plating efficiency} = \frac{a \times 100}{b}$$

where a = number of colonies

b = number of cells plated

$$\text{Surviving fraction} = \frac{c}{d}$$

where c = plating efficiency of treated cells

d = plating efficiency of untreated control cells

$$\text{Dose enhancement ratio} = \frac{e}{f}$$

where e = the concentration of the cytotoxic drug that produces a certain surviving fraction

f = the concentration of the cytotoxic drug, that in combination with DFMO (at a concentration of 10 mM) produces the same surviving fraction as the cytotoxic drug alone.

$$e > f$$

$$\text{Dose modification ratio} = \frac{f}{e}$$

where e and f are as above, $f > e$.

3.6 POLYAMINES AS REGULATORS OF CELL GROWTH - RESULTS AND DISCUSSION

3.6.1 Effects of Polyamine Depletion on Cell Growth (I)

When ELD cells were stimulated to grow polyamine synthesis increased markedly, as shown by activation of ODC (I) and SAMDC (Fig. 11). The cellular decarboxylase activities and polyamine contents were at their highest levels during the period of most rapid growth. As the cells progressed into plateau phase and the rate of cell proliferation decreased, the ODC and SAMDC activities and polyamine contents returned to their basal levels.

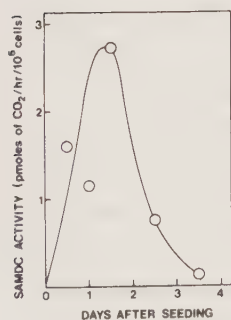


Figure 11. The S-adenosylmethionine decarboxylase (SAMDC) activity of ELD cells stimulated to grow by dilution in fresh medium.

When ELD cells were treated with 5 mM DFMO, cell proliferation was markedly inhibited (I). There was a continuous decrease in growth rate, beginning approximately 1 day after seeding, i.e. when the cells

had traversed more than one cell cycle. After 4 days of DFMO treatment the cell number was 38% lower than in cultures grown in the absence of the inhibitor.

Despite the fact that DFMO is an irreversible inhibitor of ODC, the inhibition of enzyme activity was not complete (I). This may be due partly to the high rate of synthesis of the enzyme (59, 60) and partly to the increased half-life of ODC observed in DFMO-treated cells (Table 1). Notably, during plateau phase, when ODC was nondetectable in control cells, there was a residual enzyme activity in DFMO-treated cells.

Table 1. Effects of ornithine decarboxylase inhibitors on the half-life of the enzyme in ELD cells.

Treatment ^a	$t_{\frac{1}{2}}^b$	
	min	(%)
Control	21	(100)
DFMO	75	(357)
MO	102	(486)

^a Cells were seeded at a density of 1×10^5 cells/ml and were cultured in the absence or presence of 5 mM α -difluoromethylornithine (DFMO) or 5 mM α -methylornithine (MO). At 24 hr of growth, cycloheximide (50 μ g/ml) was added to the suspension cultures and samples were then removed at 5 min intervals. The cells were pelleted and analysed for ornithine decarboxylase (ODC) activity.

^b ODC activity was plotted against time of cycloheximide treatment and the rate of decrease in activity, i.e. the half-life ($t_{\frac{1}{2}}$), was estimated by linear regression analysis.

DFMO treatment caused a rapid and permanent depletion of putrescine and spermidine (I). In contrast, there was a significant increase in cellular spermine content. This observation may be explained by the fact that putrescine normally inhibits spermine synthase by competing with spermidine (Section 2.4.3, Fig. 4). Since the putrescine content is reduced to extremely low levels in DFMO-treated cells, the putrescine-mediated inhibition of spermine synthase is relieved. Thus, spermidine competes more favorably for the catalytic site of spermine synthase, resulting in increased spermine formation (and a decreased spermidine content).

That there was no inhibition of spermine synthesis by DFMO-treatment was clearly demonstrated in an experiment described by Mamont et al. (179). They showed that the incorporation of ^3H -ornithine into putrescine and spermidine was reduced by 98% in DFMO-treated rat hepatoma cells, whereas the incorporation into spermine was unchanged.

DFMO has been shown to affect the polyamine content and the proliferation of other cell types in a similar manner (124, 180-184). Despite the fact that essentially the same DFMO concentrations were used, the time course of polyamine depletion varied somewhat among cell lines. This appears to be due to their different basal polyamine levels. The delay in onset of DFMO-induced growth retardation appears to be related to the size of the polyamine pools, i.e. the higher the basal polyamine levels, the longer the delay. In fact, when DFMO was added to ELD cells after 23 hr of growth (i.e. after the initial surge of putrescine and spermidine synthesis) growth proceeded as in untreated control cells (I). Thus, when the cells had been permitted to expand their polyamine pools, during the course of cell growth, they were strongly resistant to the action of DFMO. Interestingly, when DFMO was added at 23 hr after seeding, ODC was only transiently decreased. Even when a 5-fold higher DFMO concentration (25 mM) was added (at 23 hr after seeding), the same phenomenon was found and cell proliferation was completely unaffected (unpublished results).

This finding suggests that cells with high levels of ODC activity and polyamines, may be resistant to the action of DFMO. Several DFMO resistant cell variants have been recently isolated (180, 185, 186). These cells exhibit elevated levels of ODC and putrescine, when compared to the wild type cells.

Figure 12 shows the effects of DFMO treatment on the cell cycle traverse of ELD cells. In control cultures, the cells accumulated in G1 when grown to a plateau phase. When treated with 5 mM DFMO, however, the fraction of cells in S increased. Thus, polyamine depletion appears to block ELD cells in the S phase and/or to slow their rate of progression through this phase.

It has been suggested that the interference with DNA synthesis observed in polyamine-depleted cells is the result of a decrease in initiation frequency and/or in the rate of DNA chain elongation (27). It thus seems as if polyamines may stimulate DNA replication by increasing the initiation frequency and/or by participating as cofactors

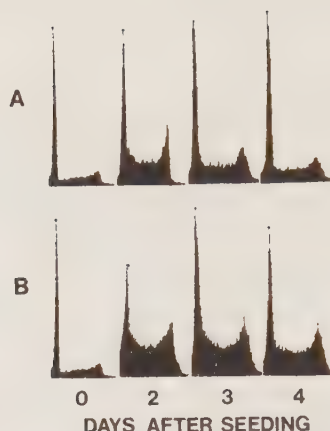


Figure 12. Effect of α -difluoromethylornithine (DFMO) treatment on the cell cycle distribution of ELD cells. The cells were grown in the absence or presence of 5 mM DFMO. The DNA distributions were determined by flow cytometric analysis. The y- and x-axes of the individual DNA histograms represent number of cell nuclei and nuclear DNA content, respectively, and are scaled arbitrarily. The DNA distributions show two peaks with relative DNA contents of 1.0 and 2.0, respectively; the first peak represents cell nuclei with a 2C DNA content (G1 phase) and the second peak represents cell nuclei with a 4C DNA content (G2 phase). The region between the 2C and the 4C peaks represents cell nuclei synthesizing DNA (S phase). Panel A. Untreated control cells; Panel B. DFMO-treated cells.

in the DNA synthetic reaction.

Although MO specifically inactivates ODC, ELD cell proliferation was not affected by 5 mM MO treatment (I). This was attributed to the fact that the putrescine concentration decreased only transiently and that the spermidine content was not markedly affected. MO caused a marked increase in cellular spermine content, probably by the same mechanism as did DFMO. Thus, during the period of MO-induced putrescine deprivation, spermidine competed more favorably for the active site of spermine synthase and as a consequence the spermine content increased.

MO treatment resulted in a substantially increased ODC activity, as revealed by the *in vitro* assay technique. This accumulation of ODC may be explained by the fact that MO stabilizes the enzyme, as revealed by an increase in its half-life (Table I). In the *in vitro* assay, there is a 700-fold dilution step, which implies that the inhibitor concentra-

tion in the reaction mixture amounted to 8 μM at the most. Since MO is a reversible inhibitor (K_i 20-40 μM) (Section 2.5.1) this dilution relieved the inhibition that occurred in the cell and liberated the accumulated amount of enzyme. The high ODC activity (elevated by as much as 30-fold) may explain why polyamines were not significantly depleted. The amount of MO available in the cell may not have been able to suppress all this ODC activity.

Despite the lack of an inhibitory effect on ELD cell growth, MO (at a 5 mM concentration) markedly inhibited the growth of rat hepatoma cells (123, 124) and mouse L1210 cells (124). A possible explanation for this is that the putrescine and spermidine content was considerably higher in ELD cells than in the other cells. Thus, when rat hepatoma and mouse L1210 cells were treated with 5 mM MO, their putrescine and spermidine content was indeed depleted. This was not the case in ELD cells. Accordingly, a clone of the rat hepatoma cells, with markedly increased putrescine and spermidine levels compared to the parental cell, showed partial resistance to the growth inhibitory effect of MO treatment (180).

MO does not effectively inhibit tumor cell growth *in vivo*. DFMO, however, has been found to suppress tumor growth, when administered in the drinking water of animals (187, 188). DFMO was also found to markedly decrease normal prostatic growth in rats (189). In all the systems used, DFMO treatment was shown to significantly decrease the putrescine levels and, to some extent, the spermidine levels. The spermine levels, however, were unaffected.

In conclusion, the experiments with DFMO and MO clearly demonstrate the superiority of the former ODC inhibitor in depleting cellular polyamines and in inhibiting cell proliferation. The results show that DFMO-induced polyamine depletion precedes growth inhibition. Nevertheless, a normal growth rate is maintained for a limited time period even in the absence of polyamine synthesis. Once the polyamine content has been sufficiently depleted, however, cell growth is suppressed and cells accumulate in the S phase of the cell cycle. Thus, polyamines, at least putrescine and spermidine, seem to be essential for optimal rates of DNA synthesis and cell growth.

3.6.2 Reversal of DFMO-Induced Growth Inhibition (II)

An obvious means of determining whether polyamines are absolutely

required for cell growth and division, is to add polyamines to the culture medium of cells inhibited in their growth by polyamine depletion. Thus, putrescine- and spermidine-depleted ELD cells (grown in the presence of 5 mM DFMO for 72 hr) were reseeded at a low cell density in DFMO-containing (5 mM) medium, and were supplemented with putrescine, 1,3-diaminopropane (a putrescine homolog) or Mg^{2+} .

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-containing medium. When putrescine- and spermidine-depleted cells were reseeded in DFMO-containing medium, they temporarily increased their growth rate, but stopped growing after about one doubling. This increase in growth rate may partly be attributable to the presence of small amounts of polyamines in the medium, derived from the fetal calf serum supplement (190). Accordingly, Harada et al. (191) found that putrescine- and spermidine-depleted Chinese hamster ovary cells grew faster in medium containing undialyzed serum than in medium containing dialyzed serum.

Surprisingly, the ODC activity increased slightly after reseeding in DFMO-containing medium and the enzyme activity was never totally eradicated. This stimulation of putrescine synthesis may also partly contribute to the slight increase in growth rate.

Despite the presence of DFMO, the intracellular spermine content increased after reseeding. Since the putrescine concentration is very low it does not exert any inhibition on spermine synthase. Thus, any putrescine and spermidine that becomes available, as a result of residual ODC activity and/or addition as a medium component, will be converted into spermine.

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-free medium. When putrescine- and spermidine-depleted cells were reseeded in DFMO-free medium, the ODC activity increased precipitously, but not to quite the same level as in control cells. A residual amount of DFMO probably accounted for this difference. Both putrescine and spermidine reached maximum levels at 36 hr after reseeding, approximately 24 hr later than in control cells. The resumption of cell proliferation showed a temporal correlation to the increases in cellular putrescine and spermidine contents. Thus, an optimal rate of cell proliferation was reached after a delay of about 12 hr. Similar results were obtained for putrescine- and spermidine-depleted 9L cells reseeded in DFMO-free medium (182).

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-containing medium supplemented with putrescine. When putrescine- and spermidine-depleted cells were reseeded in DFMO-containing medium supplemented with 1 mM putrescine, the putrescine and spermidine pools were rapidly replenished and normal growth was promptly restored. Thus, putrescine was taken up from the medium by the cells and converted into spermidine. That normal proliferation was restored without a delay in these cells, may be due to a faster replenishment of the polyamine pools upon addition, than upon endogenous production. In fact, since DFMO was present in the medium, polyamine addition was the only means of restoring polyamine levels. The residual ODC activity was inhibited by the addition of 1 mM putrescine, probably because putrescine induces the formation or release of ODC-antizyme (Section 2.4.2, Fig. 4), a protein that binds to and reversibly inactivates ODC.

Mamont et al. (179) found that the effect of putrescine was dose dependent. A maximum rate of cell proliferation was achieved at a 10 μ M concentration. However, a normal growth rate was not restored at any of the putrescine concentrations used (0.1 μ M to 1 mM). We have not used putrescine concentrations lower than 1 mM for reversal experiments, since normal growth was indeed restored and no adverse effects were seen at this concentration (II).

Mamont et al. (179) also showed that addition of spermidine (10 μ M) and spermine (1 μ M) to rat hepatoma cells, previously incubated for 3 days in DFMO-containing medium, nearly restored maximum rates of cell proliferation. Moreover, Porter and Bergeron (192) recently showed that a number of putrescine and spermidine analogs prevented DFMO-induced growth inhibition in L1210 cells to varying degrees.

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-containing medium supplemented with 1,3-diaminopropane. When putrescine- and spermidine-depleted cells were reseeded in DFMO-containing medium supplemented with 5 mM 1,3-diaminopropane, they temporarily increased their growth rate, but only to the same extent as did cells reseeded in DFMO-containing medium without supplements. 1,3-Diaminopropane inhibited the ODC activity that was resistant to DFMO treatment. The mechanism of action of 1,3-diaminopropane involves the formation or release of ODC antizyme (Fig. 4) (65) and possibly also a prevention of ODC gene transcription (99). The spermine content decreased in 1,3-diaminopropane-supplemented cells, reflecting the inhibition that this

lower putrescine homolog exerts on spermine synthase (Section 2.5.1, Fig. 4).

Porter and Bergeron (192) found that 1,3-diaminopropane could not prevent DFMO-induced growth inhibition in L1210 cells. In contrast, Alhonen-Hongisto et al. (193) found that DFMO-induced inhibition of ^3H -thymidine and ^{14}C -leucine incorporation, disappeared upon addition of 3 mM 1,3-diaminopropane. Similarly, Mamont et al. (179) found that 1 mM 1,3-diaminopropane, when added to DFMO-treated rat hepatoma cells, re-stored cell proliferation equally well as did 10 μM putrescine. Why this non-physiological amine may take over the function(s) of the polyamines under certain circumstances remains to be elucidated.

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-containing medium supplemented with Mg^{2+} . The involvement of polyamines in various steps of protein synthesis is well established. Thus, polyamines have been shown to increase the rate of aminoacyl-tRNA formation (194), the fidelity of translation (194), the initiation of protein synthesis (195, 196) and the rate of polypeptide chain elongation (197). In many of these reactions, the polyamines can be replaced by Mg^{2+} , but only at much higher concentrations. In the case of DFMO-induced growth inhibition, however, Mg^{2+} (at 1-20 mM concentrations) was unable to replace putrescine.

In conclusion, DFMO-induced growth inhibition was only reversed under conditions that resulted in replenishment of the putrescine and spermidine levels. Neither 1,3-diaminopropane nor Mg^{2+} had the capacity to reverse growth inhibition caused by putrescine and spermidine deficiency. The slight stimulation of growth observed, when putrescine- and spermidine-depleted cells were reseeded in DFMO-containing medium was attributed to polyamine uptake from the medium in combination with a low rate of polyamine synthesis. These studies strongly suggest that there is an absolute requirement of polyamines for optimal cell growth and division.

3.6.3 Effects of DFMO Treatment on Cellular Ribonucleotide and Deoxyribonucleotide Levels (III)

When ELD cells are stimulated to grow, ODC (I) and SAMDC (Fig. 11) are activated as previously mentioned. Although the cellular putrescine concentration increases markedly, the concentration of deSAM may not increase, simply because deSAM is rapidly utilized in spermidine and

spermine synthesis. The normal cellular deSAM levels are very low, when compared to the polyamine levels. In exponentially growing ELD cells (72 hr after seeding), the deSAM content was less than 0.2 pmol/ 10^6 cells, while those of putrescine, spermidine and spermine were 0.16, 1.29 and 0.88 nmol/ 10^6 cells, respectively (III).

When cells are treated with DFMO, the ODC activity is inhibited (I), but the SAMDC activity increases to levels much higher than those of growth-stimulated cells (179). The increased SAMDC activity may be a consequence of depletion of spermidine, which supposedly acts as a repressor of SAMDC (Section 2.4.3). Since the aminopropyl transferase reactions are not taking place to any appreciable extent in DFMO-treated cells (putrescine- and spermidine-depleted), the deSAM formed in the SAMDC reaction will accumulate in the cell. In ELD cells treated with 5 mM DFMO for 72 hr, the deSAM level was more than 1500 times higher than in untreated control cells (III). Similar findings have been made in other cell types (189, 198-200). The SAM levels, however, were not affected by DFMO-treatment (III).

Since the aminopropyl transferase reactions do not take place to any appreciable extent in DFMO-treated cells, the accompanying MTA production is substantially decreased. In addition, high concentrations of deSAM have been found to inhibit spermidine synthase (Section 2.4.3, Fig. 4). As previously mentioned (Section 2.4.4, Fig. 3), MTA is rapidly cleaved by a phosphorylase, yielding adenine and 5-methylthioribose 1-phosphate, which are both reutilized. In the absence of polyamine synthesis, this reutilization cannot take place, and the accumulation of deSAM may represent a loss of both adenine and methionine for the cell. This raised the question, whether part of the inhibitory effect on DNA synthesis and growth observed in polyamine-depleted ELD cells (I, II) might be attributable to a block in the recycling of adenine, i.e. a decrease in the adenine ribonucleotide pools. In fact, ethionine is known to decrease the hepatic ATP content by trapping the adenine moiety in the form of S-adenosylethionine (201).

Analysis of acid extracts of DFMO-treated (5 mM for 72 hr) ELD cells rendered surprising results (III). The levels of all ribonucleotides identified (UMP, UDP, UTP, CMP, CDP, CTP, AMP, ADP and ATP) were elevated in DFMO-treated cells. The total adenine nucleotide pool was 2.35 times higher in DFMO-treated cells than in untreated control cells; the major contributors to the increase being ATP and ADP. Due to the

greater increase in ATP than in ADP and AMP, the energy charge was slightly elevated as compared to untreated control cells. The mechanism(s) behind this increase in cellular ribonucleotide pools in DFMO-treated cells remains to be elucidated.

A preliminary observation shows that the concentrations of the ribonucleotides increase slightly (except for that of UTP which decreases) during density-dependent inhibition of ELD cell growth (SM Oredsson, M Kanje and O Heby; unpublished results). This indicates that the increase in ribonucleotide content occurring during DFMO-induced growth inhibition may partly be due to a decreased utilization of ribonucleotides for nucleic acid synthesis. Accordingly, the ATP content and the total adenine nucleotide content was found to exhibit a negative correlation with the growth rate of a series of rat hepatomas (202).

An interesting possibility is that part of the antiproliferative effect of polyamine depletion may be attributable to the accumulation of ATP and ADP. High ATP levels, as well as high ATP/ADP ratios have been reported to markedly reduce the rate of DNA synthesis, both in intact cells and in isolated nuclei (203-205). It has recently been shown that cultured cells (except for a normal human intestine cell line), when treated with agents that produce an increase in the cellular ATP pool, are inhibited in their DNA replication (206, 207). In fact, treatment of human tumor cells with ADP or ATP has been found to block cells in the S phase of the cell cycle (206). As shown in Fig. 12, ELD cells accumulate in the S phase as a result of polyamine depletion.

DNA synthesis requires an equal supply of the four deoxyribonucleotides dATP, dCTP, dGTP and dTTP (208). The synthesis of deoxyribonucleotides is catalyzed by ribonucleotide reductase (Fig. 13) (208, 209). There is a general agreement that ribonucleotide reductase is the rate-limiting enzyme in DNA synthesis (209). Ribonucleotide reductase is stimulated by ATP and is inhibited by dATP. The inhibition caused by dATP results in a rapid depletion of the deoxyribonucleotide pools (208, 209). The turnover of the dGTP pool appears to be particularly rapid, since the size of this pool only suffices for about 30 seconds of DNA synthesis (208). When the deoxyribonucleotide pools are depleted (as a result of inhibition of ribonucleotide reductase), DNA replication ceases. Hunting et al. (210) have recently shown that an increase in the cellular ATP content results in a corresponding increase in dATP.

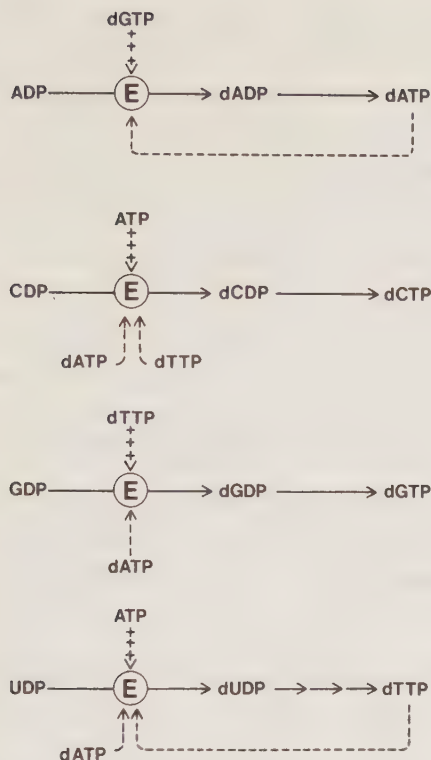


Figure 13. Regulation of deoxyribonucleotide synthesis. E, ribonucleotide reductase; +++, stimulation; ---, inhibition.

Therefore, our working hypothesis has been that the high ATP pool present in DFMO-treated cells, causes an increase in the dATP pool, which inhibits the ribonucleotide reductase activity and consequently DNA synthesis.

A recent analysis of the deoxyribonucleotide pools in DFMO-treated ELD cells rendered unexpected results (B Nicander, SM Oredsson and O Heby; unpublished results). The dATP, dCTP, dGTP and dTTP pools were low in slowly proliferating ELD cells, but increased several-fold upon growth stimulation. In control cells, there was then a progressive decrease in the deoxyribonucleotide pools during a 4-day period, reflecting the decreasing growth rate of the cells. In DFMO-treated cells, however, the deoxyribonucleotide pools remained at elevated levels. After 72 hr of DFMO treatment, the deoxyribonucleotide contents were about twice as high in DFMO-treated cells as in control cell. Nevertheless, the ratios between the individual deoxyribonucleotides (dATP:dTTP:dCTP:dGTP) were similar (Table 2).

These results are intriguing in view of the fact that the ribo-

Table 2. Effects of α -difluoromethylornithine treatment on the deoxyribonucleotide content of ELD cells.

Treatment ^a	dATP ^b	dTTP ^b	dCTP ^b	dGTP ^b
	pmoles/10 ⁶ cells			
Control	7 (175) ^c	19 (475)	8 (200)	4 (100)
DFMO	17 (243)	36 (514)	20 (286)	7 (100)

^a Cells were seeded at a density of 1×10^5 cells/ml and cultured for 72 hr in the absence or presence of 5 mM α -difluoromethylornithine (DFMO).

^b These analyses were performed by Björn Nicander at the Department of Biochemistry I, Karolinska Institutet, Stockholm.

^c Figures in parenthesis show the content of each deoxyribonucleotide in relation to that of dGTP (%).

nucleotide reductase activity, and the deoxyribonucleotide pool sizes, appear to reflect the growth rate of cells (209). When cells in culture are stimulated to synthesize DNA, the pools increase in size before the onset of DNA replication, and reach a maximum during S phase (208). In Ehrlich ascites tumor cells grown *in vivo* the level of ribonucleotide reductase activity was shown to parallel a decrease in growth fraction (211). Moreover, the rate of ³H-thymidine incorporation into DNA was found to parallel the ribonucleotide reductase activity (211). Despite the fact that DFMO-treated ELD cells grew very slowly (I), the deoxyribonucleotide pools were elevated. Therefore, there appears to be an uncoupling between the ribonucleotide reductase activity and the DNA polymerase activity as a result of polyamine depletion. Available deoxyribonucleotides are apparently not utilized for DNA synthesis.

In conclusion, DFMO treatment resulted in elevated levels of ribonucleotides and deoxyribonucleotides and there was no apparent imbalance among the various pools. Therefore, it seems unlikely that DFMO-induced inhibition of DNA synthesis is a result of depletion of or imbalance in these pools. The fact that the deoxyribonucleotide contents are elevated in polyamine-depleted cells, despite a low rate of DNA synthesis, may instead suggest a role for the polyamines in the DNA synthetic machinery.

3.6.4 Ultrastructural Changes in DFMO-Treated Cells (IV)

The effects of DFMO treatment on the ultrastructure of 9L cells and ELD cells were evaluated by transmission electron microscopy. In 9L cells treated with 10 mM DFMO for 72 hr, the mitochondria were 2.3 times larger than in untreated control cells. There was a slight disorganization of the mitochondrial cristae and rarefaction of the matrix. In DFMO-treated 9L cells, the putrescine and spermidine content was reduced by 95%, while the spermine level was unaffected. When 1 mM putrescine was added to the culture medium after 48 hr of DFMO treatment, and the cells examined 24 hr later, the mitochondria were similar to those of untreated 9L cells. In these cells, the putrescine, spermidine and spermine levels were 77, 91 and 108%, respectively, of those in control cells. Thus, DFMO-induced enlargement of mitochondria was reversed when the intracellular polyamine levels were replenished, implicating polyamines in the maintenance of mitochondrial integrity. In contrast to 9L cells, ELD cells did not exhibit any major effects on mitochondrial morphology as a result of DFMO treatment (Fig. 14 A).

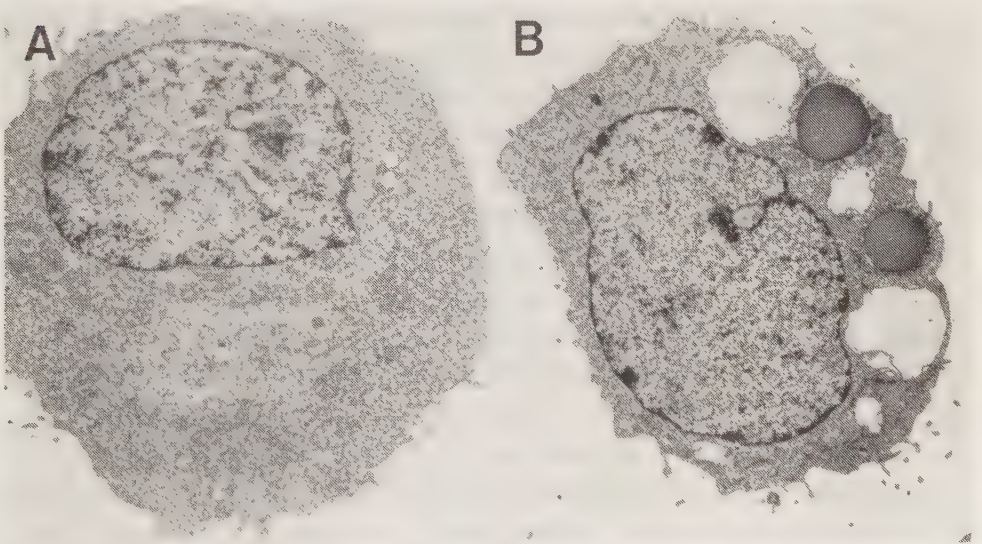


Figure 14. Effects of α -difluoromethylornithine (DFMO) and methylglyoxal-bis(guanylhydrazone) (MGBG) treatment on the ultrastructure of ELD cells. Cells were seeded at a density of 1×10^5 cells/ml and cultured for 36 hr in the presence of 5 mM DFMO (A) or 10 μ M MGBG (B). The ultrastructure of DFMO-treated cells does not differ significantly from that of untreated control cells. Magnification 7700X.

Because MGBG treatment is known to cause extensive mitochondrial damage (100, 114), 9L cells were treated with this polyamine synthesis inhibitor in order to compare its effects with those of DFMO. Rather than being extensively damaged, the mitochondria of MGBG-treated 9L cells (80 μ M for 48 hr) were only slightly enlarged. They were 1.3 times larger than those of control cells. On the other hand, when ELD cells were treated with 10 μ M MGBG the classical effects of this polyamine synthesis inhibitor on mitochondria were found (Fig. 14 B). Thus, the mitochondria were 3-4 times larger than in control cells. The matrix appeared flocculent and was considerably less dense than the surrounding cytoplasm. The swollen mitochondria contained dense granules and membraneous myelin figures which are presumably degenerating inner membranes. The damage appeared to render the organelles nonfunctional. Accordingly, Porter et al. (212) showed that the ATP pools were depleted in MGBG-treated cells.

Pyruvate utilization, a measure of mitochondrial function (174) was studied in MGBG and DFMO-treated 9L cells (IV). At 72 hr of MGBG (80 μ M) and DFMO (10 mM) treatment, the pyruvate utilization was 34 and 56%, respectively, of that in control cells. Although MGBG caused less mitochondrial enlargement than did DFMO, the inhibition of mitochondrial function was more pronounced in the MGBG-treated 9L cells. When the intracellular polyamine levels were replenished, pyruvate utilization was largely restored.

Polyamines have been shown to stabilize isolated mitochondria against spontaneous and induced swelling (19, 213, 214). Whether polyamines have a stabilizing effect on mitochondria in the cell is unknown. It has been suggested that polyamines exhibit a non-specific affinity for the negatively charged phospholipid groups and sialic acid residues associated with cells and organelles (19, 214). The resultant charge neutralization may produce a membrane that is less permeable and mechanically stronger. In addition, since there is a negative potential across the inner mitochondrial membrane, the polyamines could be drawn to this membrane (214). In fact, polyamines have been proposed to slow mitochondrial respiration by binding to the inner mitochondrial membrane and by displacing K^+ (214). The exchange of K^+ for protons across the inner mitochondrial membrane is known to be a critical event during mitochondrial respiration (215). Polyamine depletion may increase respiration and possibly also ATP production by allowing for increased

binding of K^+ to the inner mitochondrial membrane. In preliminary observations we have found that respiration is increased in DFMO-treated ELD cells (B Löwkvist and SM Oredsson; unpublished results) and we have also shown that the cellular ATP pools are elevated (III). On the other hand, respiration was decreased in DFMO-treated 9L cells as evaluated by pyruvate utilization (IV).

In DFMO-treated 9L cells there was a definite increase in the number of cytoplasmic as well as membrane-associated ribosomes (IV). When putrescine was added to the culture medium of DFMO-treated cells, to replenish intracellular polyamine levels, the normal ribosome distribution was restored. In MGBG-treated 9L cells, there was a slight increase in the number of cytoplasmic ribosomes. In contrast to 9L cells, ELD cells did not exhibit any effects on the ribosome distribution as a result of MGBG and DFMO treatment. The mechanism(s) behind the effect of DFMO and MGBG on the ribosome distribution in 9L cells remain to be elucidated.

In conclusion, the changes in mitochondrial structure and function and ribosomal distribution observed in DFMO-treated 9L cells appear to be related to polyamine depletion. All the effects observed were reversed when putrescine and spermidine pools were restored by the addition of putrescine to the culture medium. DFMO treatment has not been observed to cause mitochondrial enlargement in any other cells including ELD cells. MGBG treatment on the other hand is known to cause extensive mitochondrial swelling, as observed in ELD cells. Therefore, it was surprising to find that mitochondria were only slightly enlarged in MGBG-treated 9L cells. The mechanism(s) behind these differences remain to be elucidated.

3.7 POLYAMINES AS REGULATORS OF CELL DIFFERENTIATION - RESULTS AND DISCUSSION (V)

Undifferentiated F9 cells grow in culture as tightly packed colonies exhibiting the typical appearance of embryonal carcinoma cells. The cells are rounded and have a high nuclear:cytoplasmic ratio. When F9 cells were growth-stimulated by trypsinization and change of medium, polyamine synthesis increased. During the exponential phase of growth, the population doubling time was approximately 9 hr. Rapid growth was characterized by a very short G1 phase, and the majority of cells were in the S phase.

When F9 cells were treated with 5 mM DFMO, growth came to a complete arrest 2 days after plating and the cells accumulated in the G1 phase of the cell cycle. Putrescine and spermidine were nondetectable 1 day after plating, whereas the spermine content was increased. Within 3 days of DFMO treatment, the cells moved apart and the colonies became less compact. The cells gradually changed their morphology to triangular and finally giant, flat cells. In the giant cells, most of the organelles were sequestered in the perinuclear region and the nuclear:cytoplasmic ratio was markedly reduced.

Because retinoic acid is known to induce embryonal carcinoma cell differentiation (158), the F9 cells were treated with this inducer in order to compare its effects with those of DFMO. Retinoic acid treatment (1 μ M for 2 days) resulted in growth inhibition and G1 accumulation of the F9 cells. The changes in polyamine contents were similar to those of untreated control cells. Within 2 days of retinoic acid treatment, there was a change in cell morphology similar to that of DFMO-treated cells. Based on morphological criteria, F9 embryonal carcinoma cells differentiated into endoderm-like cells.

The retinoic acid- and DFMO-induced morphological changes were paralleled by induction of plasminogen activator activity. α -Feto-protein (as determined by indirect immunofluorescence) could not be detected in the differentiated cells (R Carlsson, SM Oredsson and O Heby; unpublished results). These morphological and biochemical changes suggest that F9 embryonal carcinoma cells differentiate into parietal endoderm cells (158, 216).

Putrescine (50 μ M) added together with retinoic acid did not prevent differentiation. When putrescine was added together with DFMO, however, differentiation was completely prevented. This implies that retinoic acid and DFMO induce F9 cell differentiation by different mechanisms. Thus, even though retinoic acid is known to inhibit ODC (217-220), the differentiation induced by retinoic acid is not a direct result of putrescine (and spermidine) depletion. When putrescine was added to cells previously incubated with DFMO for two days, differentiation was also prevented. However, when putrescine was added at 4 days of DFMO treatment or later, the cells were already terminally differentiated, and the process could not be reversed.

The present data indicate that inhibition of ODC activity and the resulting polyamine deficiency progressively induces a state of

differentiation where the F9 cells, which were initially highly malignant, have no further proliferative potential. Similar results have been obtained for two other embryonal carcinoma cell lines (221, 222), a neuroblastoma (223) and a melanoma (224) cell line. In these cells, polyamines are required for optimal rates of cell proliferation, and when growth is inhibited by polyamine depletion, differentiation is induced. Thus, polyamines seem to act as negative effectors of cell differentiation in these systems.

DFMO treatment of human HL-60 promyelocytic leukemia cells resulted in growth inhibition without concomitant induction of differentiation (221, 225, 226). When DFMO was added together with an inducer, such as retinoic acid, dimethyl sulfoxide or a phorbol ester, differentiation proceeded unaffected.

When a variety of normal cells were stimulated to differentiate, ODC activity was induced and the polyamine content increased (227-231). Inhibition of the ODC activity by DFMO treatment inhibited this differentiation. Moreover, the DFMO-mediated inhibition of differentiation was prevented by addition of polyamines to the culture medium. In these normal cells, polyamines appear to act as positive effectors of differentiation.

The level of DNA methylation is presently believed to be a key element in the control of gene function and differentiation (232). Actively transcribed genes are presumably undermethylated. SAM is a substrate in DNA methylation and in many other transmethylation reactions. Since DFMO treatment causes an elevation of deSAM to levels that are higher than those of SAM (Section 3.6.3), it may be important to determine whether deSAM may compete with SAM in DNA methylation reactions, thus possibly affecting differentiation. In fact, it has been shown that deSAM acts as a weak inhibitor of histamine, acetylserotonine, homocysteine and noradrenaline methyltransferases (233, 234).

The complex nature of differentiation is demonstrated by the various responses to DFMO treatment. Thus, polyamines may act as positive or negative effectors. In the case of F9 embryonal carcinoma cells (and some other tumor cell lines), the polyamines appear to act as negative effectors. Thus, DFMO-mediated polyamine depletion induces differentiation of these highly malignant cells, to cells without proliferative capacity. Because polyamine synthesis inhibitors are generally antiproliferative in action, and in addition induce terminal

differentiation of certain highly malignant cancer types, they may be particularly useful in the treatment of these neoplastic diseases.

3.8 POLYAMINE SYNTHESIS INHIBITORS AS ANTICANCER AGENTS - RESULTS AND DISCUSSION

3.8.1 Effects of DFMO-Induced Polyamine Depletion on Alkylation- and Carbamoylation-Induced Cytotoxicity (VI)

Because of their growth inhibitory effects, polyamine synthesis inhibitors may be used alone as anticancer agents. In addition, since polyamines exert a stabilizing effect on DNA, polyamine depletion may affect the DNA structure and render it more susceptible to cytotoxic damage. Accordingly, Hung et al. (235) recently found that the cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Table 3), was significantly increased in polyamine-depleted 9L cells. Nitrosoureas, such as BCNU, decompose under physiological conditions to yield isocyanates (which carbamoylate) and carbonium ions (which alkylate) (Fig. 15) (236, 237). Based on this knowledge of nitrosourea decomposition, a study was designed to evaluate the effects of polyamine depletion on alkyla-

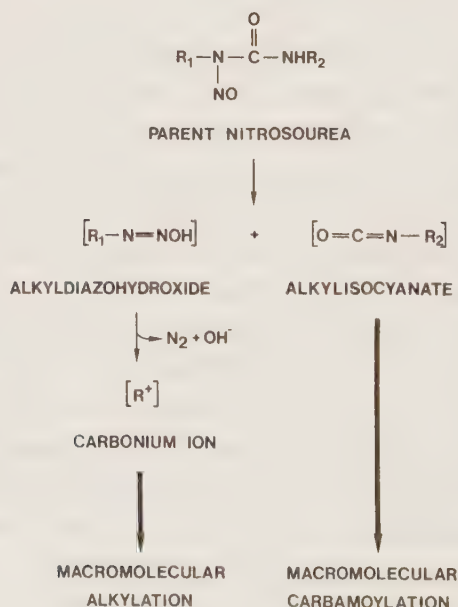
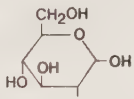


Figure 15. A simplified scheme for the decomposition of nitrosoureas.

tion- and carbamoylation-induced cytotoxicity. Four nitrosoureas possessing varying degrees of alkylating and carbamoylating activities were used.

Table 3. Molecular structures and properties of the nitrosoureas studied. Data for alkylating and carbamoylating activities and for decomposition time are taken from a paper of Wheeler (237).

	$\begin{array}{c} \text{O} \\ \parallel \\ \text{R}_1-\text{N}-\text{C}-\text{NHR}_2 \\ \\ \text{NO} \end{array}$		RELATIVE TO BCNU		
	STRUCTURE R ₁	R ₂	ALKYLATING ACTIVITY	CARBAMOYLATING ACTIVITY	DECOMPOSITION TIME
BCNU	ClCH ₂ CH ₂ —	—CH ₂ CH ₂ Cl	1	1	1
CHLOROZOTOCIN	ClCH ₂ CH ₂ —		1.70	0.03	0.49
BHCNU			0*	128*	0.13*
ENU	CH ₃ CH ₂ —	—H	—	—	—
MNU	CH ₃ —	—H	0.26	0.38	0.16

*The values given for alkylating and carbamoylating activities and decomposition time are those of 1,3-bis(cyclohexyl)-1-nitrosourea (lacks the OH groups on the cyclohexyl derivatives). The values for BHCNU are similar.

Mechanisms of action of the nitrosoureas studied (VI). 2-(3-(2-Chloroethyl)-3-nitrosoureido)-D-glycopyranose (chlorozotocin) (Table 3), a chloroethylnitrosourea, is a bifunctional alkylating agent, which lacks carbamoylating activity (237). It decomposes to form chloroethylcarbonium ions, which may form cross-links between DNA strands and between DNA and protein. The reaction has been suggested to take place in two steps (Fig. 16) (238). The first step is assumed to be chloroethylation of a nucleophilic site in one strand of DNA. The second step involves displacement of Cl[−] by a nucleophilic site in the opposite DNA strand, or in a protein, resulting in an ethyl bridge formation. The DNA-DNA cross-links seem to be more important than the

DNA-protein cross-links in mediating cytotoxicity (239). Because of intramolecular inactivation of the isocyanate decomposition product, chlorozotocin lacks carbamoylating activity (240, 241).

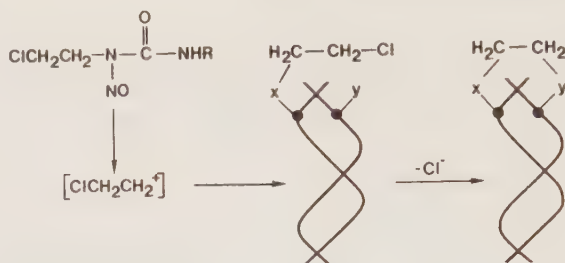


Figure 16. A schematic representation of the proposed mechanism of DNA cross-linking by chloroethylnitrosoureas. X and Y are nucleophilic sites on opposite DNA strands.

1,3-Bis(*trans*-4-hydroxycyclohexyl)-1-nitrosourea (BHCNU) is a carbamoylating agent, which completely lacks alkylating activity (Table 3) (237). The isocyanate decomposition product undergoes carbamoylation reactions with α -amino acids, with the terminal amino groups of peptides and proteins and with the ϵ -amino groups of lysine moieties (237, 242). Carbamoylation of nucleic acids is negligible (237, 243, 244). Although the extent of protein carbamoylation is considerable, the role of this event in nitrosourea-induced cytotoxicity is poorly understood (242).

N-Methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea (ENU) are monofunctional alkylating agents, which cause methylation and ethylation, respectively, of DNA (Table 3) (237, 242). These agents cannot cause cross-link formation. MNU and ENU alkylate similar nucleophilic sites in DNA, but there are striking differences in the relative abundance of alkylated nucleosides (201). ENU has a greater reactivity for base and phosphate oxygens than does MNU (201). Both agents have the capacity to carbamoylate (242), but to a much lesser degree than BHCNU (Table 3).

Effects of polyamine depletion on the cytotoxicity of the nitrosoureas (VI). Polyamine-depleted 9L cells (treated with 10 mM DFMO for 48 or 72 hr) were exposed to chlorozotocin, BHCNU, MNU or ENU for 1 hr. A colony forming efficiency assay was used to evaluate drug-induced cell kill. The plating efficiency of untreated control cells was about 70%. DFMO treatment alone did not affect the plating efficiency of 9L

cells, implying that DFMO treatment *per se* was not cytotoxic. The 4 nitrosoureas showed considerably different degrees of cytotoxicity. At a 10% surviving fraction, the doses used for chlorozotocin, BHCNU, MNU and ENU were 10 μ M, 250 μ M, 1.6 mM and 7.6 mM, respectively.

The cytotoxicity of chlorozotocin, a bifunctional alkylating agent, was significantly increased in polyamine depleted 9L cells. The dose enhancement ratio of 1.3 was the same as that found for BCNU (235). When putrescine was added to the DFMO-treated cultures, to replenish intracellular polyamine levels before chlorozotocin treatment, the potentiation phenomenon was prevented.

The cytotoxicity of BHCNU, a carbamoylating agent, was significantly decreased in polyamine-depleted 9L cells. This effect could be partially prevented by the addition of putrescine (before BHCNU treatment), which restored the intracellular polyamine levels. It should be mentioned, that the DFMO-induced decrease in carbamoylation-induced cytotoxicity occurred at much higher cytotoxic drug concentrations than did the DFMO-induced potentiation of alkylation-induced cytotoxicity. Polyamine depletion did not affect the cytotoxicity of the monofunctional alkylating agents MNU and ENU.

These studies show that DFMO-induced polyamine depletion only potentiates the cytotoxicity caused by bifunctional alkylation, while the cytotoxicity caused by monofunctional alkylation is unaffected. Accordingly, the initial step in cross-link formation, i.e. the chloroethylation of DNA (Fig. 16), is not increased in polyamine-depleted cells (245). It appears that polyamine depletion affects the DNA structure, thus facilitating cross-link formation (Fig. 16). Hung et al. (246) recently found that polyamine depletion indeed alters the DNA conformation of 9L cells, as measured by viscoelastometry. The mechanism for the DFMO-induced decrease in carbamoylation-induced cytotoxicity remains to be elucidated.

The DFMO-induced potentiation of chloroethylnitrosourea (e.g. chlorozotocin and BCNU)-induced cytotoxicity appears to be a general phenomenon for this class of drugs (235, 245). Not only DFMO treatment, but also MGBG treatment increases chloroethylnitrosourea cytotoxicity (247). Potentiation of chloroethylnitrosourea-induced cytotoxicity by DFMO has also been demonstrated *in vivo* (248).

In conclusion, cytotoxicity induced by bifunctional alkylating agents was significantly increased in polyamine-depleted 9L cells,

while cytotoxicity induced by monofunctional alkylation was unaffected. Because the cytotoxicity of bifunctional alkylating agents is supposed to be the result of DNA cross-link formation, it appears that polyamine depletion potentiates this process by changing the DNA structure.

3.8.2 Effects of DFM0-Induced Polyamine Depletion on the Cytotoxicity of *cis*-Diamminedichloroplatinum(II) (VII)

As described in the previous section (3.8.1), polyamine depletion alters the DNA structure to facilitate cross-link formation induced by bifunctional alkylating agents (VI). To further elucidate the interaction between polyamines and DNA and to investigate whether polyamine depletion may interfere with the action of other DNA damaging agents, the cytotoxicity of *cis*-diamminedichloroplatinum(II) (*cis*-platinum) was evaluated in polyamine-depleted 9L cells.

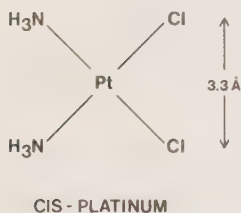


Figure 17. The molecular structure of *cis*-diamminedichloroplatinum(II) (*cis*-platinum).

Mechanism of action of *cis*-platinum. *Cis*-platinum, which is a square planar platinum coordination complex (Fig. 17), interacts with the bases of DNA, either monofunctionally (in one strand) or bifunctionally (in one strand or between opposite strands) (Fig. 18) (249-251). Cross-links between DNA and protein are also formed. However, cytotoxicity induced by *cis*-platinum appears to be the result of bifunctional cross-link formation in DNA (252-255).

The two Cl atoms of *cis*-platinum, are separated by a distance of 3.3 Å (256), which is close to the interplanar base distance (3.4 Å) in B DNA (136). There are a number of nucleophilic groups in DNA, i.e. amino groups and other reactive sites of the bases, which are separated by a distance of 3.4 Å (257). Presumably, *cis*-platinum-DNA complexes are formed when these nucleophilic groups displace the leaving ligands (the two Cl atoms of *cis*-platinum). Either intra- or interstrand cross-links may form.

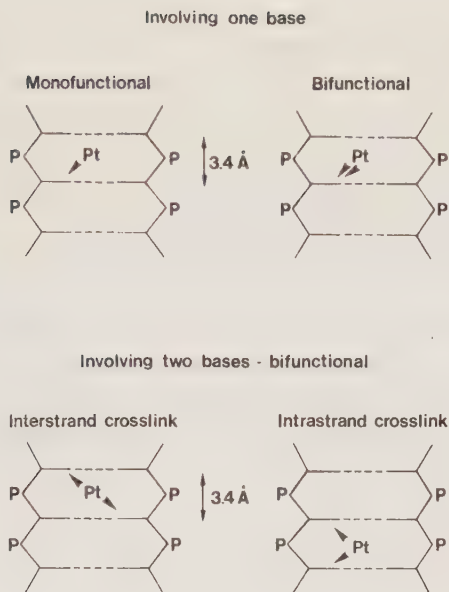


Figure 18. A schematic representation of the proposed binding modes of *cis*-diamminedichloroplatinum(II) (Pt) to DNA.

Effects of polyamine depletion on the cytotoxicity of *cis*-platinum (VII). Polyamine-depleted 9L cells (treated with 10 mM DFMO for 72 hr) were exposed to *cis*-platinum for 1 hr. A colony forming efficiency assay was used to evaluate drug-induced cell kill. Surprisingly, the cytotoxicity of *cis*-platinum was significantly decreased in polyamine-depleted 9L cells. At 10, 1 and 0.1% surviving fractions, the dose modification factor was about 2. The decrease in cytotoxicity could be prevented by adding putrescine (which replenished the intracellular polyamine levels) prior to *cis*-platinum treatment. The inhibitory effect of polyamine depletion on *cis*-platinum cytotoxicity has been confirmed, using the sister chromatid exchange assay (which measures chromosomal damage) and the alkaline elution assay (which measures DNA cross-link formation) (258,259).

In conclusion, it appears that polyamine depletion alters the DNA structure and thereby changes the distance between nucleophilic groups, making cross-linking with *cis*-platinum mechanistically and thermodynamically unfavourable. Thus, an alteration in DNA conformation by polyamine depletion may confer a therapeutic advantage for certain agents (chloroethylnitrosoureas, VI) and a therapeutic dis-

advantage for others (*cis*-platinum).

3.8.3 Effects of DFMO-Induced Polyamine Depletion on the Cytotoxicity of an S Phase Specific Agent (VIII)

Rupniak and Paul (260) and Sunkara et al. (261) showed that polyamine synthesis inhibition caused an accumulation of normal cells in the G1 phase, and of transformed cells in the S phase. Consequently, if one uses polyamine synthesis inhibitors to accumulate tumor cells in the S phase, one should then be able to give S phase specific agents and kill tumor cells selectively. At the same time, normal cells should be protected because of their arrest in G1. To further evaluate this possibility, polyamine-depleted 9L cells were subjected to treatment with 1- β -D-arabinofuranosylcytosine (ara-C) (an S phase specific drug).

Mechanism of action of ara-C. Ara-C treatment supposedly kills cells that are synthesizing DNA. Ara-C may interfere with DNA synthesis in many different ways, e.g. 1) by inhibiting ribonucleotide reductase (262), 2) by competing with dCTP for the active site of DNA polymerase (263), and 3) by inhibiting DNA polymerase when already incorporated into DNA (264). In addition to being cytotoxic to cells in S phase, ara-C inhibits the entry of cells into this phase (265-267). Thus, when treating cells with ara-C, the S phase cells are killed, while cells in the G1, G2 and M phases continue cycling at their normal rates until they reach the G1-S boundary, where they become arrested. This block is reversible within 6 hr of treatment.

Effects of polyamine depletion on the cytotoxicity of ara-C (VIII). Polyamine-depleted 9L cells (treated with 10 mM DFMO for 72 hr) were exposed to ara-C for 24 hr. A colony forming efficiency assay was used to evaluate drug-induced cell kill. Ara-C exerted considerably less cytotoxicity on DFMO-treated than on untreated control cells. When putrescine was added to the DFMO-treated cells, to replenish the intracellular polyamine levels, ara-C treatment exerted the same cytotoxicity as on control cells.

These results are in apparent conflict with those obtained by Sunkara et al. (260, 268), which showed that ara-C cytotoxicity was significantly increased in DFMO-treated HeLa cells. These differences may be explained by the fact that 9L cells and HeLa cells respond differently to DFMO treatment. Thus, HeLa cells accumulate in the S phase, while 9L cells do not accumulate in any particular phase, but instead

slow their progression through all phases of the cell cycle.

Cell kinetic studies showed that 9L cells, treated with ara-C for 6 hr and then released from the ara-C block by refeeding with ara-C-free medium, traversed the S phase in a semisynchronous fashion (VIII). Thus, a large fraction of cells were blocked at the G1-S border. However, this phenomenon was not observed when polyamine-depleted 9L cells were treated with ara-C for 6 hr, and then refed with ara-C-free and DFMO-free medium. In DFMO-treated cultures, the rate of cell cycle progression was apparently so low that only a few cells reached the G1-S boundary during the 6 hr ara-C treatment period. This low rate of cell cycle progression may partly explain the decreased cytotoxicity of ara-C in polyamine-depleted cells. Thus, when the rate of DNA synthesis is reduced, ara-C cytotoxicity may be counteracted. When intracellular polyamine levels were replenished by addition of putrescine, a normal rate of cell cycle progression was resumed, and the cell cycle perturbations induced by ara-C were virtually identical to those found for cells treated with ara-C alone.

These studies show that transformed cells are not necessarily arrested in the S phase as a result of polyamine depletion, as was previously postulated (260, 268). This is further demonstrated by the fact that highly malignant F9 embryonal carcinoma cells were arrested in the G1 phase when treated with DFMO (V). ELD cells, on the other hand, tended to accumulate in the S phase as a result of polyamine depletion (Fig. 12). Because of the different responses to DFMO treatment among transformed cells, the cytotoxicity of an S phase specific drug may not always be potentiated. It also remains to be established whether normal cells are always blocked in G1 by polyamine depletion as postulated (260, 261).

3.9 Summary

The role of the polyamines in cell growth and division has been studied by analyzing the effects of polyamine depletion. Thus, Ehrlich ascites tumor (ELD) cells were treated with α -difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC). As a result, the cellular putrescine and spermidine content decreased and there was a marked reduction in growth rate. The growth-inhibited ELD cells accumulated in the S phase of the cell cycle. Normal growth was restored under conditions that resulted in replenishment of the intracellular polyamine levels, i.e. when polyamines were added

to the growth medium or when DFMO was removed. These studies strongly suggest that cells have an absolute requirement of polyamines for optimal growth and division.

Endogenous adenine is almost exclusively derived from 5'-methylthioadenosine, a by-product of the spermidine and spermine synthase reactions. Even though these reactions do not take place to any appreciable extent in DFMO-treated cells, there was a significant increase in adenine ribonucleotide and deoxyribonucleotide pools in ELD cells, as a result of DFMO treatment. The fact that all ribonucleotide and deoxyribonucleotide pools were elevated and that there was no major imbalance between individual nucleotides, suggests that DFMO treatment slows the rate of DNA synthesis by interfering with the DNA synthetic machinery rather than with the precursor pools.

To determine whether polyamine depletion is associated with morphological changes, ELD cells and 9L rat brain tumor cells were treated with DFMO and studied by transmission electron microscopy. The ELD cell morphology was unaffected by DFMO treatment. In contrast, DFMO-treated 9L cells exhibited significantly enlarged mitochondria, which were functionally impaired as evaluated by pyruvate utilization. These mitochondrial changes appear to be the result of polyamine deficiency, since they were reversed when polyamine pools were replenished by the addition of putrescine.

The role of polyamines in differentiation was evaluated by using F9 mouse embryonal carcinoma cells, an *in vitro* model of mammalian embryogenesis. When F9 cells were depleted of their polyamines by DFMO treatment, they accumulated in the G1 phase of the cell cycle and were induced to differentiate. Putrescine administration completely prevented the DFMO-induced differentiation. When comparing the multiple phenotypic changes induced by DFMO with those induced by retinoic acid, they were clearly similar. These changes, which include morphological alterations and elevated levels of plasminogen activator, and the fact that α -fetoprotein was not synthesized, suggest that F9 cells differentiate into parietal endoderm.

Because of their growth inhibitory effects, and because of their capacity to induce terminal differentiation of certain malignant cell types, DFMO and other polyamine synthesis inhibitors may have clinical utility. Since polyamine depletion appears to change the DNA conformation, polyamine synthesis inhibitors were analyzed for their capacity

to modify the response of cancer chemotherapeutic agents that exert their cytotoxicity by damaging DNA. The cytotoxicity of chlorozotocin, a bifunctional alkylating agent, was significantly increased, the cytotoxicities of N-methyl-N-nitrosourea and N-ethyl-N-nitrosourea, monofunctional alkylating agents, were unaffected and the cytotoxicity of *cis*-platinum was significantly decreased in polyamine-depleted 9L cells. Thus, the alteration in DNA conformation, caused by polyamine depletion, may modulate the therapeutic effect of cytotoxic agents in a positive or negative direction.

It has been postulated that polyamine depletion blocks normal cells in the G1 phase and transformed cells in S phase of the cell cycle. Therefore, polyamine-depleted 9L cells were treated with an S phase specific agent, 1- β -D-arabinofuranosylcytosine (ara-C). The cytotoxicity of ara-C was not potentiated, but instead reduced in DFMO-treated 9L cells. This finding may be explained by the fact that 9L cells did not accumulate in the S phase when treated with DFMO. Instead their progression through all the phases of the cell cycle was slowed. It is thus apparent that DFMO treatment does not consistently block transformed cells in the S phase. This was further demonstrated by the fact that the highly malignant F9 embryonal carcinoma cells were arrested in the G1 phase when treated with DFMO.

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ERRATUM

The following paragraph was omitted in the typing of page 38:

Chromomycin A3-stained cells were analyzed using the Lawrence Livermore National Laboratory flow cytometer (VIII). This flow cytometer was adjusted to 458 nm (200 mW power output) for excitation. Fluorescence emission was measured through a Corning Model 3-71 spectral filter (passing wavelengths longer than 500 nm). The emission wavelength of chromomycin A3 is 580 nm. A 256-channel histogram, showing the distributions of fluorescence among the cells in the population being analyzed, was generated for each sample. The percentages of cells in each phase of the cell cycle were derived from the DNA distributions as described by Dean (170).

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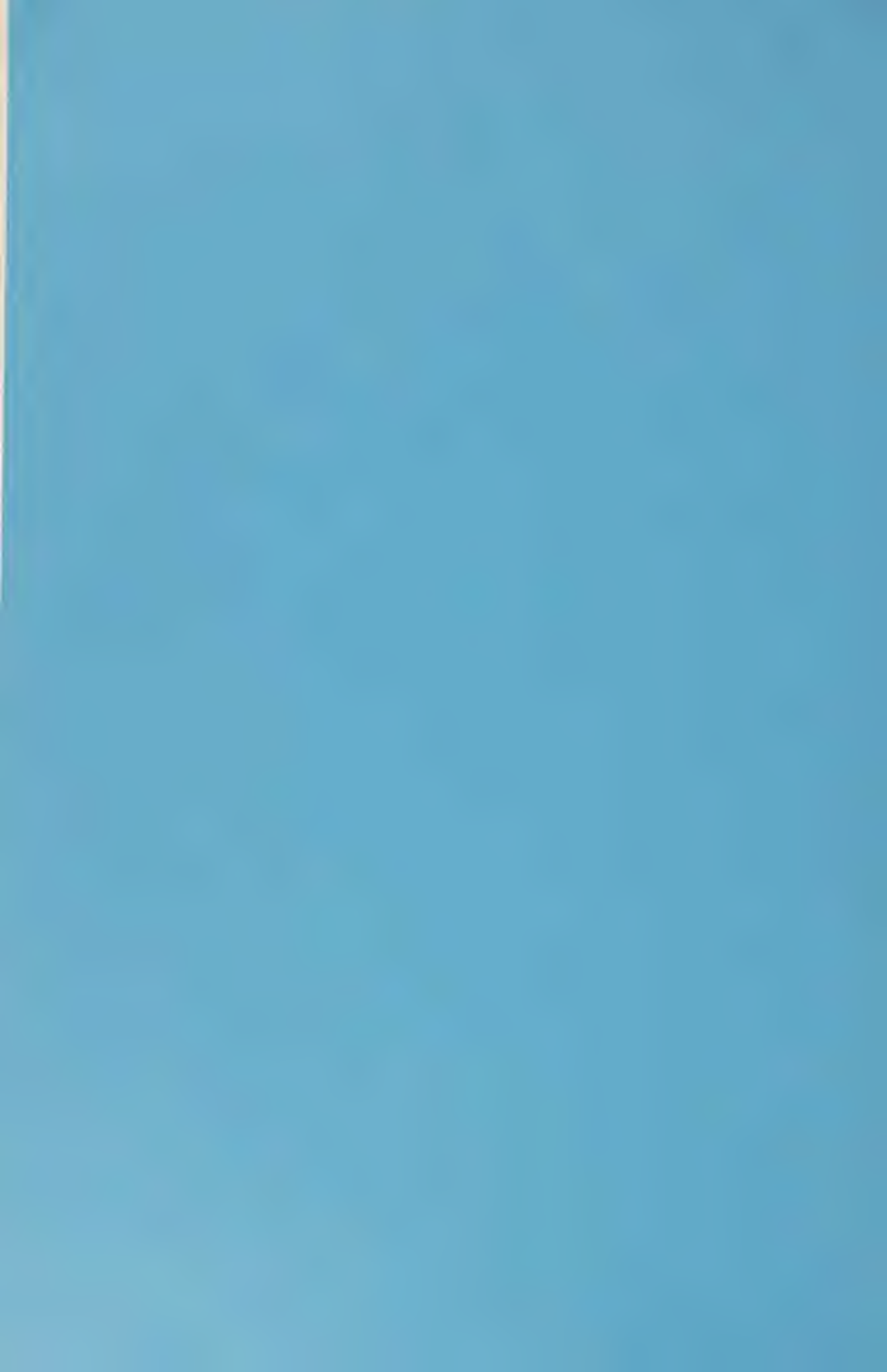
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REVERSAL OF THE GROWTH INHIBITORY EFFECT OF α -DIFLUOROMETHYLORNITHINE
BY PUTRESCINE BUT NOT BY OTHER DIVALENT CATIONS

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SUMMARY

Treatment with α -difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC), depletes the putrescine and spermidine content, and reduces the growth rate of Ehrlich ascites tumor cells.

The addition of putrescine, which is the immediate precursor of spermidine, promptly replenished the intracellular putrescine and spermidine pools and completely reversed the antiproliferative effect of DFMO. A sequential accumulation of spermine, spermidine and putrescine was observed.

1,3-Diaminopropane, a lower homolog of putrescine, did not reverse the antiproliferative effect of DFMO, despite its structural similarity and identical positive charge. By inhibiting remaining ODC activity, resistant to 5 mM DFMO, and possibly by inhibiting spermine synthase activity, 1,3-diaminopropane produced a further decrease in total polyamine content by reducing the spermine content.

Mg^{2+} , which can replace putrescine in many *in vitro* reactions, completely lacked the capacity to reverse the antiproliferative effect of putrescine and spermidine deficiency.

INTRODUCTION

The recent development of inhibitors that specifically interfere with enzymes of the polyamine biosynthetic pathway, has made it possible to deplete cells of their polyamines (1, 2). It has been shown that polyamine depletion invariably reduces the rate of cell growth and proliferation (1-4). The most efficient and most widely used polyamine synthesis inhibitor is α -difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC) (L-ornithine carboxy-lyase; EC 4.1.1.17) (5). The growth inhibitory effect of DFMO-induced polyamine depletion has been demonstrated in a variety of cell systems (1-4). The reduction in the rate of cell proliferation follows putrescine and spermidine depletion and is usually not apparent until the cells have traversed one or two cell cycles (6, 7). This suggests that the cells contain enough polyamines to maintain a normal growth rate for a limited time period. Once the polyamine content has been sufficiently depleted, however, macromolecular synthesis and cell growth become suppressed. The specificity of DFMO is clearly demonstrated by the fact that the inhibitor-induced cessation of cell proliferation is reversed by supplementation of polyamines (6, 8). Thus, polyamine deficiency seems to be the actual cause of inhibition of proliferation.

As the polyamines are positively charged at a physiological pH, it may be suggested that one of their functions is to serve as endogenous cations, the concentrations of which can be regulated by intracellular synthesis and degradation. Indeed, in many of the *in vitro* reactions in which effects of polyamines have been demonstrated, the polyamines can be replaced by Mg^{2+} or other cations, but only at much higher concentrations (9-11). In addition, polyamines interact specifically with e.g. DNA (12, 13) and tRNA (14, 15).

The present study was designed to determine whether the growth inhibitory effects of DFMO treatment may be reversed by divalent cations other than putrescine. Thus, polyamine-depleted Ehrlich ascites tumor cells were seeded at a low cell density in DFMO-containing medium supplemented with various divalent cations. The results show that only putrescine, which promptly replenished the intracellular levels of putrescine and spermidine, restored a normal rate of cell proliferation.

A nonphysiological diamine and a divalent metal cation had no such capacity.

EXPERIMENTAL PROCEDURES

Cell Culture. Ehrlich ascites tumor cells were adapted to suspension growth in culture in 75 cm² tissue culture flasks. The cultures were incubated without agitation at 37°C in a water-saturated atmosphere of 5% CO₂ in air. The growth medium used (Swim's S-77) was supplemented with 11.1 mM D-glucose, 0.1 mM L-cystine, 2 mM L-glutamine, 0.1 mM sodium pyruvate, 1.8 mM CaCl₂, 17.5 mM NaHCO₃, phenol red (0.001%), penicillin (50 IU/ml)-streptomycin (50 µg/ml) mixture and finally, when the pH had been adjusted to 7.4, fetal calf serum (10%). The basic Mg²⁺ concentration in the medium was 0.83 mM. Thus in experiments where the medium was supplemented with 1, 10 or 20 mM Mg²⁺ the final concentrations were 1.83, 10.83 or 20.83 mM. The cells were routinely subcultured every 3 or 4 days by a 20-fold dilution with fresh medium (from 2x10⁶ to 1x10⁵ cells per ml). During exponential growth the cell number doubled in approximately 13 hr.

Treatment protocol. Plateau phase cells (1x10⁷) were seeded in 100 ml of growth medium or in 100 ml of growth medium containing 5 mM DFMO. At 72 hr of growth, the cells were counted, and pelleted at 500 xg for 5 min. Control cells (1x10⁷) were reseeded in 100 ml of DFMO-free growth medium. DFMO-treated cells (1x10⁷ cells in each treatment group) were reseeded in 100 ml of DFMO-free growth medium or in 100 ml of DFMO-containing growth medium with or without a supplement of putrescine (1 or 10 mM), 1,3-diaminopropane (5 mM) or Mg²⁺ (1, 10 or 20 mM MgCl₂). The various media used were prewarmed to 37°C before the start of the experiment. Stock solutions of DFMO, putrescine, 1,3-diaminopropane and Mg²⁺ were made up in fresh growth medium at a 100x concentration, adjusted to pH 7.4 and sterile filtered. The cells were originally counted in a hemocytometer, but since there was no cell clumping a Coulter counter was used for routine counts. At various times after reseeded, samples were taken for cell counting, enzyme assay and polyamine analysis. Cells intended for biochemical analyses were harvested by centrifugation at 1000 xg for 10 min at 4°C and the

cell pellets were stored at -70°C .

Polyamine analysis. The cells were disrupted by sonication in ice-cold 0.2 M perchloric acid and the homogenates were kept on ice for 1 hr. The intracellular polyamine content was determined using a thin-layer chromatographic method (16), essentially as described by Heby et al. (17).

Ornithine decarboxylase assay. The cells were disrupted by sonication in ice-cold 10 mM Tris-HCl buffer (pH 7.2) containing 0.5 mM Na_2EDTA , 5 mM dithiothreitol and 50 μM pyridoxal 5'-phosphate. The ODC activity was assayed for in the presence of saturating levels (1 mM) of L-ornithine essentially as described by Jänne and Williams-Ashman (18).

Chemicals. Growth medium components were purchased from Gibco Europe (Paisley, Scotland), 1,3-diaminopropane dihydrochloride from EGA-Chemie (Steinheim/Albuch, Federal Republic of Germany) and putrescine dihydrochloride from Fluka (Buchs, Switzerland).

DL- α -difluoromethylornithine monohydrochloride monohydrate (DFMO) was generously donated by Centre de Recherche Merrell International (Strasbourg, France).

RESULTS

Figure 1A shows the growth curves for Ehrlich ascites tumor cells seeded in the absence or presence of 5 mM DFMO. Growth inhibition became apparent between 24 and 48 hr of DFMO treatment. At 72 hr after seeding, the cell density in control and DFMO-treated (polyamine-depleted) cultures were, 1.91×10^6 and 0.90×10^6 cells per ml, respectively. The control cells were reseeded in DFMO-free medium. The polyamine-depleted cells were reseeded in DFMO-free or DFMO-containing medium, the latter with or without a supplement of 1 or 10 mM putrescine, 5 mM 1,3-diaminopropane or 1, 10 or 20 mM Mg^{2+} . In each culture, the cell number was determined at 12 hr intervals during the subsequent 96 hr experimental period.

Figure 1B, shows the growth curves for the second growth cycle. They are drawn from the appropriate end points of the first growth cycle and as if all the cells had been maintained in the second growth

cycle. When polyamine-depleted cells were reseeded in DFMO-free medium or in DFMO-containing medium supplemented with 1 mM putrescine, a normal growth pattern was obtained. In those cell cultures that did not receive putrescine, however, growth was delayed by approximately 12 hr. When polyamine-depleted cells were reseeded in DFMO-containing medium without any supplements or in DFMO-containing medium supplemented with 5 mM 1,3-diaminopropane or 1 mM Mg^{2+} , they grew very slowly, and at the most doubled their number during the 96 hr experimental period. The slight differences among the growth curves for the latter three groups are not significant, as was further substantiated when these three experiments were run in parallel (Table 1).

Figure 2 shows the effects on cell growth and polyamine synthesis and content, when polyamine-depleted cells were reseeded in DFMO-free or DFMO-containing medium. The growth data (Fig. 2A, Table 1) were discussed above (cf. Fig. 1B). At the time of reseeded there was no detectable ODC activity in control cells, but a significant activity in polyamine-depleted cells (Fig. 2B). When control and polyamine-depleted cells were reseeded in DFMO-free medium, there was a marked and rapid increase in ODC activity. When polyamine-depleted cells were reseeded in DFMO-containing medium, however, the increase in ODC activity was much less pronounced. At variance with the elimination of ODC activity that was observed 72 hr after reseeded in DFMO-free medium, there was a maintenance of significant enzyme activity after reseeded in DFMO-containing medium.

In control cells, the putrescine and spermidine content showed a marked increase after reseeded in DFMO-free medium (Figs. 2C and 2D). The cellular putrescine and spermidine contents exhibited peak levels at approximately 12 and 24 hr after reseeded. DFMO treatment was previously shown to deplete the cellular putrescine and spermidine contents within 48 hr of the first growth cycle (7) and at the time of reseeded, putrescine and spermidine were nondetectable (Figs. 2C and 2D). When these polyamine-depleted cells were reseeded in DFMO-containing medium, putrescine and spermidine remained nondetectable. However, when polyamine-depleted cells were reseeded in DFMO-free medium, the putrescine (Fig. 2C) and spermidine (Fig. 2D) contents became detectable about 24 and 12 hr after reseeded, respectively. Both putrescine and spermidine reached maximum levels at 36 hr after

reseeding, i.e. approximately 24 hr later than did control cells.

Polyamine-depleted cells reseeded in DFMO-containing medium, showed roughly the same pattern of changes in spermine content as did control cells reseeded in DFMO-free medium (Fig. 2E). A considerably greater increase in spermine content was observed in polyamine-depleted cells reseeded in the absence of DFMO. It was twice that of control cells. Notably, this entire increase in spermine content took place while there was little change in putrescine and spermidine contents.

Figure 3 shows the effects on cell growth and polyamine synthesis and content, when polyamine-depleted cells were reseeded in DFMO-containing medium supplemented with 1mM putrescine, 5 mM 1,3-diaminopropane or 1 mM Mg^{2+} . The growth data (Fig. 3A, Table 1) were discussed above (cf. Fig. 1B). Polyamine-depleted cells were also reseeded in DFMO-containing medium supplemented with 10 mM putrescine or 10-20 mM Mg^{2+} (Table 1). At the higher putrescine concentration growth was slightly inhibited. At the higher Mg^{2+} concentrations, the cells changed their characteristics and attached to the plastic substratum. To estimate the growth capacity in these Mg^{2+} -supplemented media, cells were harvested by trypsinization and counted at 4 days after reseeded. These cultures contained the same number of cells as did cultures treated with the lower concentration of Mg^{2+} (1 mM) (Table 1).

Figure 3B shows the ODC activity in polyamine-depleted cells reseeded in DFMO-containing medium supplemented with 1 mM putrescine, 5 mM 1,3-diaminopropane or 1 mM Mg^{2+} . In control cells reseeded in DFMO-free medium, the ODC activity exhibited a pattern that was basically the same as that in Fig. 2B. When polyamine-depleted cells were reseeded in DFMO-containing medium supplemented with 1 mM Mg^{2+} there were no major changes in ODC activity (Fig. 3B). The ODC activity remained at an elevated level for the entire 96-hr experimental period. When polyamine-depleted cells were reseeded in DFMO-containing medium supplemented with 1 mM putrescine or 5 mM 1,3-diaminopropane, there was a gradual reduction in ODC activity.

Figures 3C and 3D, show the putrescine and spermidine contents of polyamine-depleted cells reseeded in DFMO-containing medium, supplemented with either 1 mM putrescine, 5 mM 1,3-diaminopropane or 1 mM Mg^{2+} . In control cells reseeded in DFMO-free medium, the putrescine

and spermidine contents exhibited patterns that were basically the same as those in Fig. 2C and 2D. When polyamine-depleted cells were reseeded in DFMO-containing medium supplemented with 1 mM putrescine, the intracellular putrescine (Fig. 3C) and spermidine (Fig. 3D) contents increased from nondetectable levels, to levels that were 5- and 2-fold higher, respectively, than those of control cells at 12-24 hr after reseeding. Four days after reseeding, however, both the putrescine and spermidine contents were again lower than in control cells. When polyamine-depleted cells were reseeded in DFMO-containing medium supplemented with 5 mM 1,3-diaminopropane or 1 mM Mg^{2+} , putrescine and spermidine remained nondetectable.

In polyamine-depleted cells reseeded in DFMO-containing medium supplemented with 1 mM Mg^{2+} , the spermine content exhibited roughly the same pattern of changes as did that of control cells reseeded in DFMO-free medium (Fig. 3E). When polyamine-depleted cells were reseeded in DFMO-containing medium supplemented with 5 mM 1,3-diaminopropane the spermine content declined continuously. When polyamine-depleted cells were reseeded in DFMO-containing medium supplemented with 1 mM putrescine, the spermine level increased 2-fold at 24 hr after reseeding, as compared to the level in control cells, and then decreased to control levels.

DISCUSSION

Cells that are stimulated to grow and proliferate, synthesize polyamines at a markedly increased rate (3, 4). Prevention of this increase by treatment with specific inhibitors of polyamine synthesis invariably results in growth inhibition (1-4). Growth inhibition is not an immediate phenomenon, however, but develops gradually during the course of several cell cycles (6, 7). It thus appears, that cells contain sufficient amounts of polyamines to temporarily support their growth, even in the absence of polyamine synthesis. Cells that have ceased to grow due to polyamine depletion may be stimulated to resume a normal growth rate by the addition of polyamines to the medium (8). In the present study we have addressed the question whether there is an absolute requirement of putrescine for reversal of growth inhibition or whether other divalent cations possess a similar capacity.

Polyamine depletion by DFMO treatment. After a 72 hr period of DFMO treatment, Ehrlich ascites tumor cells proliferated at a markedly reduced rate, and the cell number was 53% lower than in cultures grown in the absence of the inhibitor (Fig. 1A). DFMO reduced the putrescine and spermidine content below the level of detection by 2 days of treatment, but failed to reduce the spermine content - even after prolonged treatment (7). This observation may explain why cell growth was not completely arrested, and will be discussed below.

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-containing medium. When putrescine- and spermidine-depleted cells were reseeded in DFMO-containing medium, they temporarily increased their growth rate. It should be emphasized, however, that the total increase in cell number over the 4-day experimental period only amounted to about 8% of that of control cells (Fig. 2A). The presence of small amounts of polyamines in the medium, derived from the fetal calf serum supplement (19), may partly account for this increase in growth rate. Accordingly, Harada et al. (20) have shown that putrescine- and spermidine-depleted Chinese hamster ovary cells grow faster in medium containing undialyzed serum than in medium containing dialyzed serum.

The increased growth rate after reseeding in DFMO-containing medium may also be related to the fact that the ODC activity increased slightly. This increase in ODC activity and the fact that the enzyme activity was never totally eradicated, despite the continued presence of the very potent inhibitor, are surprising, but may be a result of the high rate of turnover of the enzyme (21).

The increase in cellular spermine content after reseeding (Fig. 2E) is probably the combined effect of the addition of medium containing undialyzed serum and the increase in ODC activity. At normal intracellular concentrations, putrescine may inhibit spermine synthase by competing with spermidine for the active site (22). At the extremely low putrescine concentrations present in DFMO-treated cells, however, this inhibition is relieved and spermine synthesis increases until the spermidine content becomes limiting.

Another consequence of DFMO treatment is an increase in S-adenosylmethionine decarboxylase activity (23, 24). The combined effect of this increase and the lack of aminopropyl group acceptors

(putrescine and spermidine), is that the decarboxylated S-adenosylmethionine content increases dramatically (23-25). Because of the kinetics of the polyamine biosynthetic enzymes and the excessive decarboxylated S-adenosylmethionine content, any putrescine (or spermidine) that becomes available, as a result of residual ODC activity and/or addition as a medium component, will be converted into spermine. Thus, the spermine level is maintained (or even increased) at the expense of putrescine and spermidine.

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-free medium. When putrescine- and spermidine-depleted cells were reseeded in DFMO-free medium, the optimal rate of cell proliferation was reached after a certain delay (Figs. 1B and 2A). The resumption of cell proliferation showed a temporal correlation to the increases in cellular putrescine and spermidine contents, as was also shown for another cell line (26). However, these increases were preceded by a rapid and marked increase in spermine content (Fig. 2E). As stated above, this effect is probably due to the elevated decarboxylated S-adenosylmethionine content, which (in the presence of the aminopropyl transferases) effectively promotes the conversion of putrescine and spermidine into spermine until its concentration becomes limiting. In accordance with this explanation, spermidine was found to accumulate prior to putrescine (Figs. 2C and D).

The ODC activity increased precipitously upon reseeding of the putrescine- and spermidine-depleted cells in DFMO-free medium, but not to quite the same level as in control cells. A residual amount of DFMO probably accounts for this difference in ODC activity.

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-containing medium supplemented with putrescine. When polyamine-depleted cells were reseeded in DFMO-containing medium supplemented with 1 mM putrescine, the polyamine pools were rapidly replenished and normal growth was restored. The dramatic increase in spermine content may seem surprising in view of the fact that putrescine is supposed to inhibit spermine synthase (22). However, the temporal pattern of polyamine accumulation is most likely the same in these cells as in putrescine- and spermidine-depleted cells reseeded in DFMO-free medium. When putrescine is added to the medium it is rapidly taken up by the cells and converted into the higher polyamines. A sequential accumula-

tion of spermine, spermidine and putrescine may have occurred before our first time point of measurement. When the polyamine pools have been restored, putrescine may again exert an inhibitory effect on spermine synthase.

The inhibition of ODC in cells supplemented with putrescine can be partly explained by the fact that putrescine induces the formation or release of ODC-antizyme (27-30), a protein that binds to and reversibly inactivates ODC. We have observed that there is a dose dependent decrease in ODC activity and growth of Ehrlich ascites tumor cells treated with increasing concentrations of putrescine (M Linden, SM Oredsson, S Anehus and O Heby; unpublished observations). Accordingly, cells supplemented with 10 mM putrescine grew somewhat slower than did control cells (Table 1).

Mamont et al. (8) found that the reversal effect of putrescine was dose dependent, maximum cell proliferation being achieved with 10 μ M putrescine. However, normal growth was not restored at any of the putrescine concentrations used (0.1 μ M to 1 mM). In our experiments, we have not used concentrations lower than 1 mM putrescine for reversal experiments, since normal growth was indeed restored and no adverse effects were seen at this concentration. It has previously been shown that reversal of DFMO-induced growth inhibition can also be achieved by addition of spermidine or spermine (1, 2, 8).

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-containing medium supplemented with 1,3-diaminopropane. When putrescine- and spermidine-depleted cells were reseeded in DFMO-containing medium supplemented with 5 mM 1,3-diaminopropane, they temporarily increased their growth rate to the same extent that did cells reseeded in DFMO-containing medium without supplements. The presence of small amounts of polyamines in the medium, derived from the fetal calf serum supplement, may account for this increase in growth rate. These experiments indicate that 1,3-diaminopropane did not reverse or counteract the effect of DFMO.

1,3-Diaminopropane is a potent inhibitor of ODC (2, 30) and as a result the putrescine, spermidine and spermine contents decrease during the course of treatment with this lower putrescine homolog. The mechanism of action of 1,3-diaminopropane involves the formation or release of ODC antizyme (2, 30) and possibly also a prevention of

transcription of the gene (31). The decreased spermine content in 1,3-diaminopropane-supplemented cells, reflects the inhibition that this lower putrescine homolog exerts on spermine synthase by competing with spermidine as substrate.

1,3-Diaminopropane, like putrescine, has been shown to inhibit growth at millimolar concentrations (32, M Linden, SM Oredsson, S Anehus, O Heby, unpublished). Therefore, the report of Alhonen-Hongisto et al. (32) was surprising in that the inhibition of ^3H -thymidine and ^{14}C -leucine incorporation observed in the presence of 6 mM DFMO disappeared when Ehrlich ascites tumor cells received 3 mM 1,3-diaminopropane in addition to 6 mM DFMO (32). Similarly, Mamont et al. (8) found that 1 mM 1,3-diaminopropane, when added to HTC cells previously incubated for 3 days with 5 mM DFMO, restored cell proliferation equally well as did 10 μM putrescine. Why this non-physiological amine may take over the function(s) of the polyamines under certain circumstances remains to be elucidated.

Effects of reseeding putrescine- and spermidine-depleted cells in DFMO-containing medium supplemented with Mg^{2+} . When putrescine- and spermidine-depleted cells were reseeded in DFMO-containing medium supplemented with 1 mM Mg^{2+} , the increases in growth rate, ODC activity and spermine content were similar to those of cells reseeded in DFMO-containing medium without any supplements. Apparently, Mg^{2+} cannot substitute for polyamines in their capacity to reverse the growth inhibition. At higher Mg^{2+} concentrations (10-20 mM, Table 1) the characteristics of the cells changed. Cell attachment requires the presence of Mg^{2+} in the culture medium and the high Mg^{2+} concentrations in fact promoted the adhesion of cells to the substratum. Melvin and Keir (33) showed that 15 mM Mg^{2+} added to baby hamster kidney cells at the time of serum stimulation, inhibited the increase in spermidine content by 27%. Since there was no effect on cell growth, they concluded that Mg^{2+} replaces the polyamines, especially spermidine. However, these results should be evaluated in view of the fact that cells treated with DFMO proliferate normally for several cell cycles despite decreasing polyamine levels (6, 7).

Conclusion. The polyamines are positively charged at a physiological pH and in many aspects their effects resemble those of Mg^{2+} . Polyamines and Mg^{2+} are ubiquitous in nature and therefore it is diffi-

cult to determine which of them is the most important ion in any particular reaction *in vivo*. The role of Mg^{2+} in metabolic regulation has been most clearly established in glycolysis and the citric acid cycle. Although the function(s) of the polyamines are not well understood at the molecular level, recent studies have shown that their concentrations are highly regulated and that normal growth requires polyamines. In this study we have shown that Mg^{2+} cannot substitute for polyamines in reversing growth inhibition that is due to polyamine deficiency. Neither 1,3-diaminopropane, a lower putrescine homolog, was able to reverse this inhibition. Thus, growth inhibition caused by DFMO, is not merely due to the loss of cations. Instead, the reversal experiments show that there is an absolute requirement of polyamines - implicating their molecular structure rather than their charge in their interaction with other compounds of the cell.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

DFMO, α -difluoromethylornithine; ODC, ornithine decarboxylase.

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Table 1. Analysis of the capacity of putrescine, 1,3-diaminopropane and Mg^{2+} to reverse DFMO-induced inhibition of Ehrlich ascites tumor cell growth.

Supplement ^a	Cell number ($\times 10^6$)	
	84 hr ^b	96 hr ^b
None	17.4 ^c (0)	20.6 ^c (0)
1 mM putrescine	204 ^d (100)	231 ^d (100)
10 mM putrescine	191 (93) ^e	181 (76) ^e
5 mM 1,3-diaminopropane	19.1 (1) ^e	13.1 (0) ^e
1 mM Mg^{2+}	17.2 (0) ^e	20.7 (0) ^e
10 mM Mg^{2+}		20.2 (0) ^e
20 mM Mg^{2+}	19.1 (0) ^e	

^aAt time 0, 1×10^7 polyamine-depleted cells (pretreated with 5 mM DFMO for 72 hr) were reseeded in 100 ml of DFMO-containing medium without other supplements or supplemented with putrescine, 1,3-diaminopropane or Mg^{2+} at various concentrations.

^bThe number of cells was determined at 84 and 96 hr after reseeding.

^cThe number of polyamine-depleted cells after 84 and 96 hr of growth in DFMO-containing medium is designated 0% (in parenthesis).

^dThe number of polyamine-depleted cells after 84 and 96 hr of growth in DFMO-containing medium supplemented with 1 mM putrescine is designated 100% (in parenthesis).

^eThe number of polyamine-depleted cells after 84 and/or 96 hr of growth in DFMO-containing medium supplemented with various concentrations of putrescine, 1,3-diaminopropane and Mg^{2+} in percent of the difference in cell numbers between d and c.

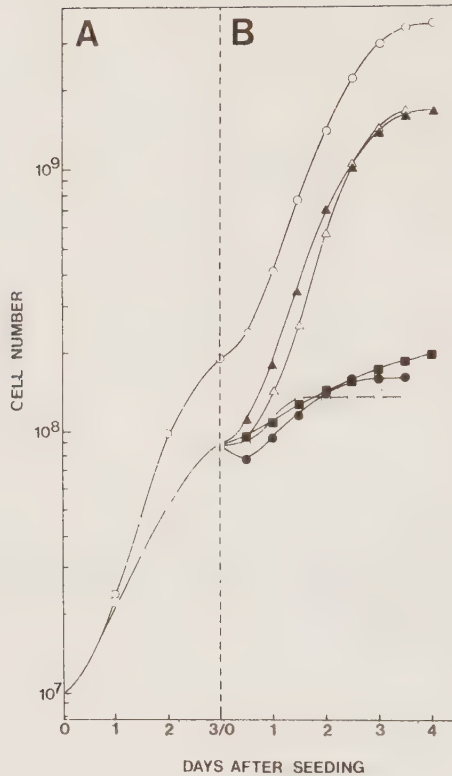


FIGURE 1. Effects of DFMO-induced polyamine depletion on growth of Ehrlich ascites tumor cells and the consequences of reseeding such polyamine-depleted cells in the presence of putrescine, 1,3-diaminopropane or Mg^{2+} . A) At time 0, 1×10^7 cells were seeded in 100 ml of growth medium in the absence (○) or presence (□) of 5 mM DFMO. B) At 72 hr after seeding the cells were harvested and reseeded at a low cell density. Control cells reseeded in DFMO-free medium (○); polyamine-depleted cells reseeded in DFMO-free (△) or DFMO-containing (5 mM) (□) medium; polyamine-depleted cells reseeded in DFMO-containing (5 mM) medium supplemented with 1 mM putrescine (▲), 5 mM 1,3-diaminopropane (●) or 1 mM Mg^{2+} (■).

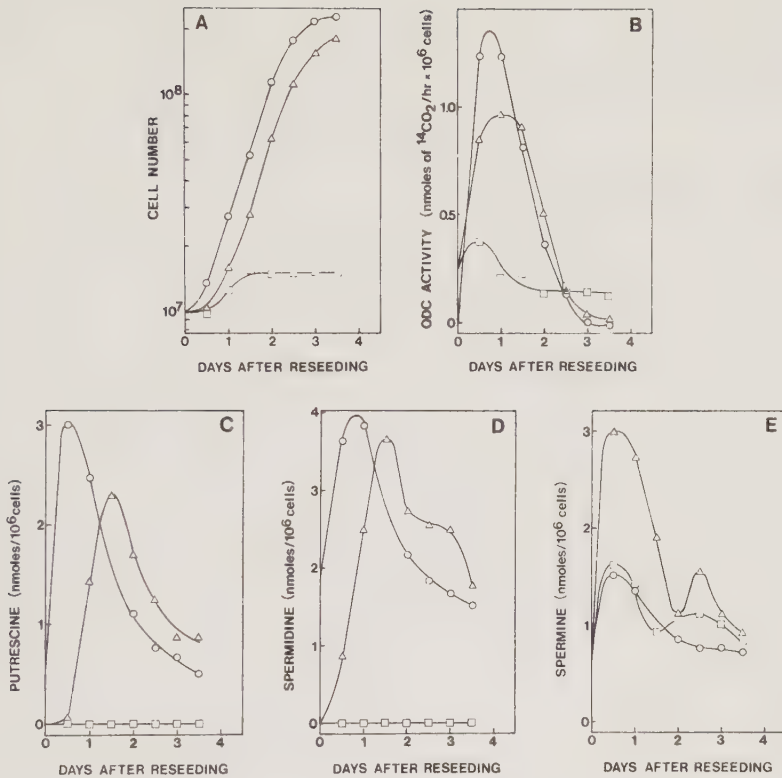


FIGURE 2. Effects of 5 mM DFMO on Ehrlich ascites tumor cell growth and polyamine metabolism. The cells were grown for 72 hr in the absence or presence of 5 mM DFMO. They were then reseeded (Day 0) at a low cell density in fresh medium containing various supplements and analyzed for their ODC activity and their putrescine, spermidine and spermine contents. Control cells reseeded in DFMO-free medium (○); polyamine-depleted cells reseeded in DFMO-free (Δ) or DFMO-containing (5 mM) (□) medium.

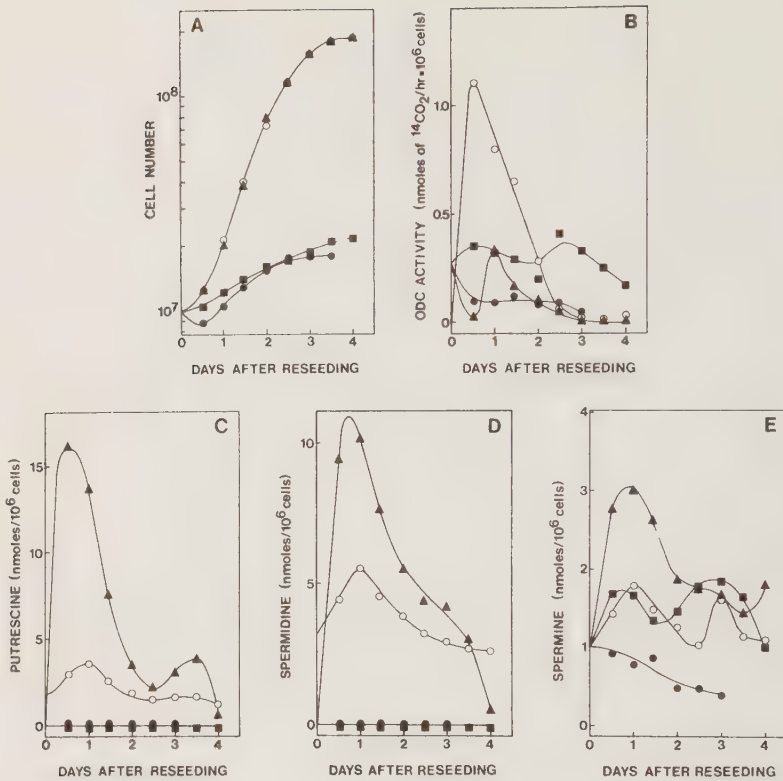
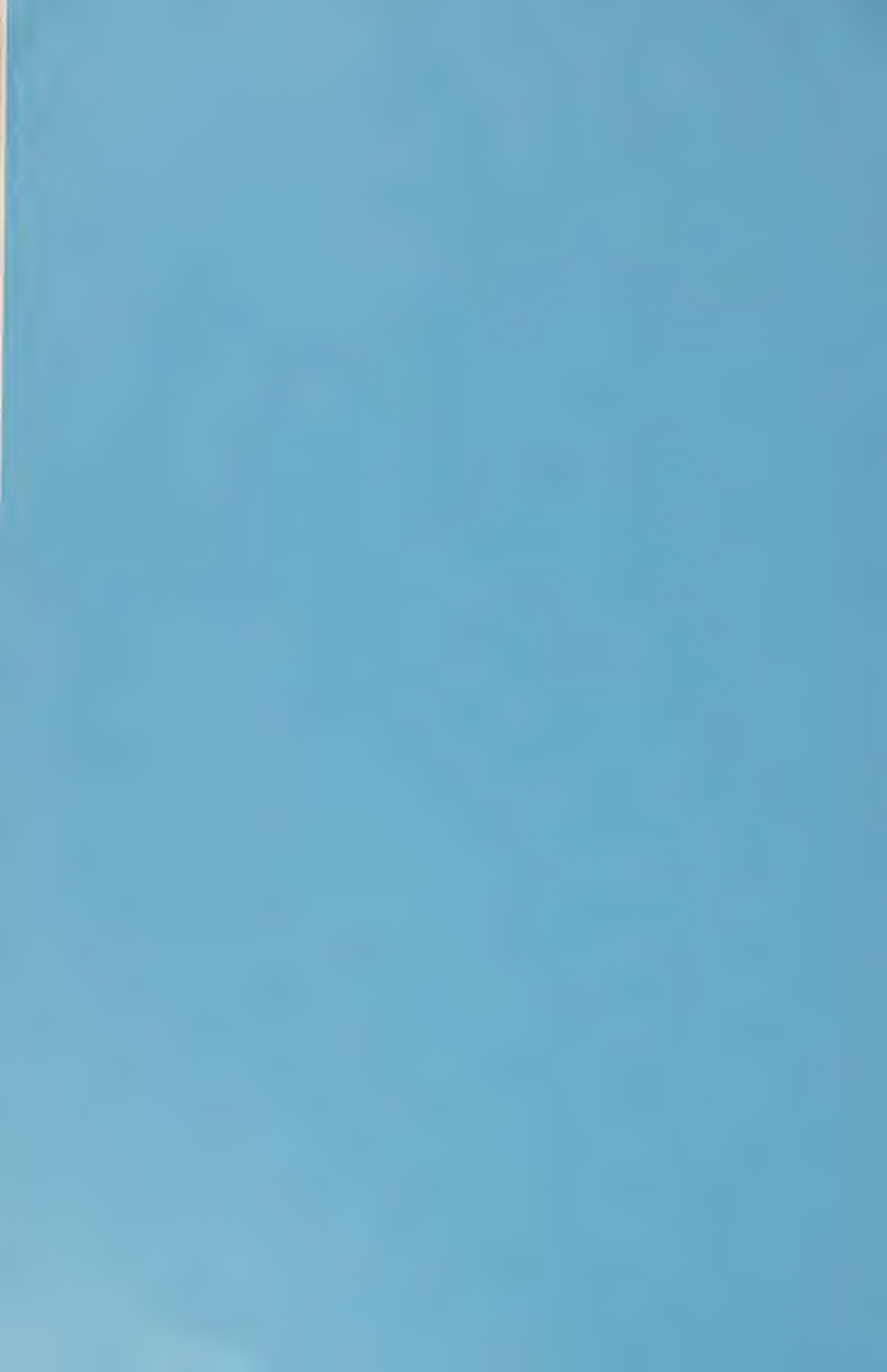


FIGURE 3. Effects of 5 mM DFMO on Ehrlich ascites tumor cell growth and polyamine metabolism. The cells were grown for 72 hr in the absence or presence of 5 mM DFMO. They were then reseeded (Day 0) at a low cell density in fresh medium containing various supplements and analyzed for their ODC activity and their putrescine, spermidine and spermine contents. Control cells reseeded in DFMO-free medium (○); polyamine-depleted cells reseeded in DFMO-containing (5 mM) medium supplemented with 7 mM putrescine (▲), 5 mM 1,3-diaminopropane (●) or 1 mM Mg^{2+} (■).

III



POLYAMINE DEPLETION INCREASES CELLULAR RIBONUCLEOTIDE LEVELS

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SUMMARY

Depletion of the putrescine and spermidine content of Ehrlich ascites tumor cells by DL- α -difluoromethylornithine treatment results in at least a 1500-fold increase in the decarboxylated S-adenosylmethionine content. The accumulation of this adenine nucleoside occurs because of the absence of putrescine and spermidine to act as amino-propyl group acceptors in the spermidine and spermine synthase reactions and because of an increase in S-adenosylmethionine decarboxylase activity. The fact that the synthesis of decarboxylated S-adenosylmethionine continues in DL- α -difluoromethylornithine-treated cells makes the pathway an adenine trap. This prompted a study of the adenine nucleotide pools. High-performance liquid chromatography showed that the total adenine nucleotide pool increased, rather than decreased, as a result of DL- α -difluoromethylornithine treatment; the major contributors to the increase being ATP and ADP, which increased 2.6 and 1.9 times, respectively. The cellular content of other ribonucleotides increased as well, particularly that of UTP and CTP.

INTRODUCTION

Depletion of the intracellular polyamine content by the use of inhibitors that specifically interfere with enzymes in the polyamine biosynthetic pathway invariably results in growth inhibition (1-3). A majority of experiments indicate that DNA replication is more sensitive to polyamine depletion than are other events in the cell cycle (4). Addition of polyamines to the growth medium of polyamine-depleted cells reverses the inhibition of growth and proliferation (5, 6) thus implying that polyamines are necessary for these processes. As a result of polyamine depletion the intracellular levels of other metabolites in the polyamine biosynthetic pathway are affected as well. Conceivably, the antiproliferative effect caused by polyamine synthesis inhibition may be partly attributable to such changes.

Inhibition of ornithine decarboxylase (L-ornithine carboxy-lyase; EC 4.1.1.17), the initial and rate-controlling enzyme in polyamine synthesis, by DL- α -difluoromethylornithine dramatically increases the cellular content of decarboxylated S-adenosylmethionine (7-10). DL- α -difluoromethylornithine is an enzyme-activated irreversible inhibitor of ornithine decarboxylase (11). Decarboxylated S-adenosylmethionine is the aminopropyl group donor in the synthesis of spermidine and spermine from putrescine and spermidine, respectively (12). This adenine nucleoside has no other known function and is normally present at extremely low concentrations (12). The K_m s for the aminopropyl transferases, spermidine synthase (S-methyladenosylhomocysteamine:putrescine aminopropyl transferase; EC 2.5.1.16) and spermine synthase (S-methyladenosylhomocysteamine:spermidine aminopropyl transferase; EC 2.5.1.), are very low (12, 13).

The accumulation of decarboxylated S-adenosylmethionine in DL- α -difluoromethylornithine-treated cells is partly due to the absence of putrescine and spermidine to act as aminopropyl group acceptors, and partly due to an increase in the S-adenosylmethionine decarboxylase (S-adenosylmethionine carboxy-lyase; EC 4.1.1.50) activity in response to the decline in spermidine content (7). The excessive accumulation of decarboxylated S-adenosylmethionine raised the question whether some of the inhibitory effect on DNA synthesis (and growth) observed in polyamine-depleted cell populations might be attributable to a block in the recycling of adenine. Our first approach has been to study the ribonucleotide pools in Ehrlich ascites tumor cells cultured in the presence or absence of DL- α -difluoromethylornithine.

MATERIALS AND METHODS

Cell culture. A hyperdiploid subline of the Ehrlich ascites tumor was adapted to suspension growth in a 1:1 mixture of Eagle's minimum essential medium and Ham's F-12 medium (contains $1 \mu\text{M}$ putrescine) supplemented with 0.2% bovine serum albumin, penicillin (50 IU/ml) and streptomycin (50 $\mu\text{g/ml}$). The cultures were incubated without agitation at 37°C in a water-saturated atmosphere of 5% CO_2 in air. The cells were routinely subcultured every 3 or 4 days by a 20-fold dilution with fresh medium (from 2×10^6 to 1×10^5 cells/ml). For the experiments, the growth medium was made up with Ham's F-12 medium lacking putrescine. The cells could be transferred between the putrescine-free and the putrescine-containing medium without change in growth rate. At time 0, 1×10^7 plateau phase cells were suspended in 100 ml of growth medium or 100 ml of growth medium containing 5 mM DL- α -difluoromethylornithine. Before addition to the culture, DL- α -difluoromethylornithine was dissolved in phosphate-buffered saline at a 500 mM concentration, the pH adjusted to 7.4 and the solution sterile filtered. The experiment was terminated 72 h after seeding. Samples were withdrawn for cell counting in a hemocytometer, and a known number of cells was pelleted by centrifugation at $1000 \times g$ (10 min, 4°C).

Ribonucleotide analysis. Since the medium contained negligible amounts of ribonucleotides (not shown), cell pellets were not washed. Immediately after collection, the cells were disrupted by sonication for 5 sec in ice-cold 1 M perchloric acid. The homogenate was kept on ice for 10 min and the protein was then removed by centrifugation at $1000 \times g$ for 10 min at 4°C . An aliquot of the supernatant was neutralized with set volumes of 10 M KOH and 2 M KHCO_3 . The test tubes were capped and kept on ice for 1 h after which the KClO_4 -precipitates were removed by centrifugation as above. The supernatants were stored in carefully capped tubes at -70°C until analysis (less than 1 month). The ribonucleotide standards were dissolved at approximately 3 mM concentrations in 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ -buffer, pH 7.0. The exact concentrations of the ribonucleotides were determined spectrophotometrically using the extinction coefficients at pH 7.0; ATP, ADP and AMP, 15.4×10^3 ; CTP, CDP and CMP, 9.1×10^3 ; and UTP, UDP and UMP, 10.0×10^3 . The standards remained stable during storage at -70°C for at least 3 months.

The concentrations of the ribonucleotides in the cell extracts

were determined by high-performance liquid chromatography using a Varian Model 5040 pump and a Model CDS 401 integrator, and a Waters Associates Model U6K injector and a Model 440 absorbance detector. The ribonucleotides were separated at room temperature on a Polyanion SI pre-packed HR 5/5 column (Pharmacia Fine Chemicals, Uppsala, Sweden). For separation we used a 15-min linear gradient of 10 to 800 mM $(\text{NH}_4)_2\text{HPO}_4/\text{NH}_4\text{H}_2\text{PO}_4$ -buffer, pH 7.0, followed by a 5-min isocratic period using the high concentration buffer. The flow rate was 1 ml/min. The ribonucleotides were detected by their absorbance at 254 nm and were identified by the retention times obtained for the appropriate standards, injected alone or mixed with the samples. The ribonucleotides were quantitated by computer integration (and by cutting and weighing) of the area under each chromatographic peak.

Protein determination. The cellular protein content was analyzed according to the method of Lowry et al. (14).

S-Adenosylmethionine, decarboxylated S-adenosylmethionine and polyamine analysis. The cells were disrupted by sonication in 25 mM H_2SO_4 . Protein was precipitated by the addition of 0.8 M perchloric acid yielding a final concentration of 0.2 M. After incubation on ice for 15 min the samples were centrifuged at 1000 x g for 10 min at 4°C. The supernatants were decanted and stored at -70°C until analysis. High-performance liquid chromatographic procedures were used for the quantitative analysis of S-adenosylmethionine (15), decarboxylated S-adenosylmethionine (15) and polyamines (16).

Cell preparative procedure for flow cytometric analysis. Cells intended for flow cytometric analysis were pelleted at 500 x g for 5 min at room temperature, the medium was decanted and the cells were resuspended in the small volume of remaining medium. Single-cell suspensions ($1-2 \times 10^6$ cells) were fixed in 1 ml of absolute ethanol (-20°C). After fixation, the cells were treated with RNase and pepsin (17).

The resulting cell nuclei were passed through a nylon mesh with a pore size of 50 μm , and finally stained with propidium iodide (17).

Flow cytometric analysis. Propidium iodide-stained cells, at a density of about 1×10^6 cells/ml, were analyzed on a Cytofluorograph System 50-H (Ortho Instruments, Westwood, MA, USA) equipped with a 4 W argon-ion laser (Model 95, Lexel Corp., Palo Alto, CA, USA). The laser line at 488 nm (450 mW power output) was used for excitation. Fluorescence emission was detected between 625 nm and infrared. About 2×10^4 cells from each preparation were analyzed. The impulses were

sorted by a 512 multichannel analyzer and processed with a PDP 11/34 computer (Digital Equipment Corp., Maynard, MA, USA). Nuclear doublets were excluded by electronic threshold settings. The peak at approximately twice the modal channel number of G1 cell nuclei represents mainly G2 phase nuclei. Since most mitotic cells lack a nuclear membrane, they are likely to be destroyed by pepsin (18) and do not appear in the 4C DNA peak. The nuclei of late-telophase cells may separate and appear in the first (G1) peak of the histogram (18). Pepsin treatment is also likely to disperse the nuclei of multinucleate cells, which may become more frequent during the course of polyamine starvation. The percentage of cells in each phase of the cell cycle were derived from the DNA distributions essentially as described by Dean (19).

Materials. Growth components were purchased from Gibco Europe (Paisley, Scotland). Bovine serum albumin (fraction V, 96-99% pure), RNase A (type III-A, bovine pancreas) and ribonucleotides (as disodium salts) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pepsin was purchased from Merck (Darmstadt, Federal Republic of Germany) and propidium iodide from Calbiochem (Lucerne, Switzerland).

RESULTS

Table 1 shows the effect of 5 mM DL- α -difluoromethylornithine treatment on the S-adenosylmethionine, decarboxylated S-adenosylmethionine and polyamine contents of Ehrlich ascites tumor cells. DL- α -difluoromethylornithine treatment depleted the cellular putrescine and spermidine content, while the spermine content was only slightly lowered as compared to that of untreated control cells. The S-adenosylmethionine level was essentially the same in control and DL- α -difluoromethylornithine-treated cells, while a significant increase in the decarboxylated S-adenosylmethionine content was found in DL- α -difluoromethylornithine-treated cells. Decarboxylated S-adenosylmethionine was nondetectable ($< 0.2 \text{ pmol}/10^6 \text{ cells}$) in control cells but increased to $0.35 \text{ nmol}/10^6 \text{ cells}$ after 72 h of treatment with DL- α -difluoromethylornithine.

Figure 1 shows that DL- α -difluoromethylornithine treatment substantially affected the ribonucleotide pools. The levels of all the ribonucleotides identified (UMP, UDP, UTP, CMP, CDP, CTP, AMP, ADP and

ATP) were elevated in DL- α -difluoromethylornithine-treated cells, whether expressed on a protein basis (as in Fig. 1) or on a cell basis (as in Table 2).

Table 2 shows the effect of DL- α -difluoromethylornithine treatment on the cellular adenine nucleotide content. The total adenine nucleotide pool was 2.35 times higher in DL- α -difluoromethylornithine-treated cells than in control cells; the major contributors to the increase being ATP and ADP. The increase in AMP as obtained with computer integration is an overestimate due to the presence of some minor unidentified peaks with elution times slightly longer than that of AMP (Fig. 1). Notably, the unidentified peaks were more prominent in DL- α -difluoromethylornithine-treated cells than in control cells. In an attempt to obtain a more accurate value, the AMP peak was trimmed and estimated by cutting and weighing (the value shown in Table 2). Due to the greater increase in ATP than in ADP and AMP in DL- α -difluoromethylornithine-treated cells, the energy charge was slightly elevated as compared to untreated control cells.

As previously indicated, the increase in ribonucleotide content was not limited to the adenine nucleotides. In fact, there was an even greater increase in the UTP and CTP levels as a result of DL- α -difluoromethylornithine treatment. The cellular UTP and CTP contents were 0.34 nmol and 0.11 nmol/ 10^6 cells, respectively, for control cells and 1.25 nmol and 0.98 nmol/ 10^6 cells, respectively, for DL- α -difluoromethylornithine-treated cells.

Table 2 also shows that cell growth was greatly suppressed by DL- α -difluoromethylornithine, the cell number being only 37% of the control at 72 h of growth. The average cell size in the DL- α -difluoromethylornithine-treated culture was somewhat larger (28%) than in the control culture as judged by the cellular protein content. Figure 2 shows that this probably was due to a change in cell cycle distribution. Thus, the percentages of cell nuclei in G1, S and G2 were 50.0, 40.4 and 9.6, respectively, in control cells and 36.7, 49.1 and 14.2, respectively in DL- α -difluoromethylornithine-treated cells. The shift in cell cycle distribution towards a higher DNA content is not due to the appearance of binuclear cells (in G1), since the DNA histogram represents the nuclear rather than the cellular DNA content.

DISCUSSION

Inhibitors that specifically interfere with the polyamine biosynthetic enzymes have recently been synthesized (1-3). These provide a means of directly evaluating the physiological role of the polyamines. Most efficient and most widely used is DL- α -difluoromethylornithine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase (11). Using DL- α -difluoromethylornithine it has been shown that cellular polyamine depletion inhibits growth in a variety of cell systems (1-3). Since the growth inhibitory effect of DL- α -difluoromethylornithine can be reversed by supplementation of polyamines (5, 6), it appears that this inhibitor is specific and that polyamines are indeed indispensable for cell growth and proliferation. However, since the intracellular levels of other metabolites in the polyamine biosynthetic pathway are also affected by inhibition of polyamine synthesis, it is of vital importance to establish to what extent these compounds contribute to the growth inhibitory effects observed.

In accordance with previous reports (7-10), polyamine depletion by DL- α -difluoromethylornithine treatment was found to substantially increase the content of decarboxylated S-adenosylmethionine in Ehrlich ascites tumor cells (Table 1). Decarboxylated S-adenosylmethionine is the aminopropyl group donor in the synthesis of spermidine and spermine from putrescine and spermidine, respectively (12). In each of these aminopropyl group transfer reactions, a stoichiometric amount of 5'-methylthioadenosine is formed. 5'-Methylthioadenosine is then rapidly cleaved to yield adenine and 5-methylthioribose 1-phosphate (12).

In DL- α -difluoromethylornithine-treated cells there is no synthesis of the aminopropyl group acceptors putrescine and spermidine. This lack of putrescine and spermidine, in combination with the increase in S-adenosylmethionine decarboxylase activity causes an accumulation of decarboxylated S-adenosylmethionine (7). Since the aminopropyl group transfer reactions are not taking place, neither 5'-methylthioadenosine nor its cleavage products, adenine and 5-methylthioribose 1-phosphate, are generated. This is especially intriguing in view of the fact that Kamatani and Carson (29) recently showed that *de novo* synthesis of adenine derived exclusively from the cleavage of 5'-methylthioadenosine and hence from the synthesis of spermidine and spermine. Therefore, it seemed likely that DL- α -difluoromethylornithine treatment would cause a decrease in the adenine nucleotide pools. However, analysis of acid

extracts of Ehrlich ascites tumor cells treated with DL- α -difluoromethylornithine rendered the opposite results. The total adenine nucleotide content was significantly increased. Mainly ATP and ADP contributed to this increase, which in turn caused a slight elevation in energy charge. In addition to the increases in ATP and ADP there was a significant increase in UTP and CTP. The mechanism(s) behind this increase in cellular nucleotide pools in DL- α -difluoromethylornithine-treated cells remain to be elucidated.

The cellular ATP content has been shown to increase by approximately a factor of two during the cell cycle in a variety of cell systems (21-24). This implies that the concentration of ATP remains essentially constant throughout the cell cycle (21, 22). Therefore, we conclude that the accumulation of S and G2 phase cells observed in DL- α -difluoromethylornithine-treated cultures can account for but a small fraction of the increase in ribonucleotide content. If the increase in ribonucleotide content was merely due to the increase in mean cell size (cell protein) the ribonucleotide content would be only 1.28 times higher in DL- α -difluoromethylornithine-treated cells than in untreated control cells. However, the ribonucleotide level in DL- α -difluoromethylornithine-treated cells was as much as 6 times higher than the control level (in the case of CTP), even if expressed on a protein basis.

A preliminary observation shows that the ribonucleotide content of Ehrlich ascites tumor cells increases slightly (except for UTP that decreases) during density inhibition of growth (SM Oredsson, M Kanje and O Heby; unpublished observations). This indicates that the increase in ribonucleotide content occurring during DL- α -difluoromethylornithine-induced growth inhibition may partly be due to a decreased utilization of ribonucleotides for nucleic acid synthesis. Accordingly, the ATP content and the total adenine nucleotide content exhibited a negative correlation with the growth rate of a series of rat hepatomas (25).

An interesting possibility, that relates to the observed increase in cellular ADP and ATP, is that part of the antiproliferative effect of polyamine depletion may be attributable to the accumulation of these nucleotides. Several studies have demonstrated the existence of a tight correlation between cellular adenine nucleotide pools and the proliferative activities of mammalian cells in culture (26-28). High ATP levels, as well as high ATP/ADP ratios have been reported to markedly reduce the rate of DNA synthesis, both in intact cells and in isolated

nuclei (28-30). It has recently been shown that cultured cells (except for a normal human intestine cell line), when treated with agents that produce an increase in the cellular ATP pool, are inhibited in their DNA replication (31, 32). In fact, treatment of human tumor cells with ADP or ATP has been found to block cells in the S phase of the cell cycle (31). As shown in Figure 2, Ehrlich ascites tumor cells accumulate in the S phase as a result of polyamine depletion.

When comparing our data with those of Rapaport (31), it should be noted that DL- α -difluoromethylornithine-induced ATP and ADP accumulation is paralleled by an increase in the UTP content, whereas ADP and ATP accumulation achieved by exogenous addition of these nucleotides causes a decrease in the UTP content.

Our present working hypothesis is based on the elevated adenine nucleotide pools in DL- α -difluoromethylornithine-treated cells, and attempts to answer whether this elevation might exert a growth inhibitory effect by influencing ribonucleotide reduction. Ribonucleotide reductase catalyzes the rate-limiting reaction for the *de novo* synthesis of deoxyribonucleoside triphosphates (33-35). This enzyme is efficiently inhibited by dATP, which causes an imbalance in the cellular deoxyribonucleoside triphosphate pools (33-35). As a result, DNA replication, which requires a strict combination of the four deoxyribonucleoside triphosphates, ceases. Hunting et al. (36) have recently shown that an increase in the cellular ATP content results in a corresponding increase in dATP. In view of these facts, the possible involvement of an increased dATP pool in DL- α -difluoromethylornithine-induced growth inhibition should be investigated.

The excessive increase in the cellular decarboxylated S-adenosylmethionine content raises the question whether this increase may have biological effects that are unrelated to those of the polyamines (8, 37). The possibility exists that decarboxylated S-adenosylmethionine (the concentration of which is almost 3 times that of S-adenosylmethionine in DL- α -difluoromethylornithine-treated cells) may substitute for S-adenosylmethionine as a substrate or act as an inhibitor in transmethylation reactions. However, decarboxylated S-adenosylmethionine was found to exhibit both a low substrate activity and a low inhibitory activity toward a number of transmethylation enzymes (38-40). Whether decarboxylated S-adenosylmethionine affects nucleic acid methylation has not yet been studied. Since methylation of proteins and nucleic acids appears to be involved in the regulation of growth and differentiation, it is

important to determine whether elevated decarboxylated S-adenosylmethionine levels affect such methylation reactions. This information is particularly important when studying the mechanism behind DL- α -difluoromethylornithine-induced cell differentiation (41-43). Recent evidence indicates that decarboxylated S-adenosylmethionine is not responsible for the inhibitory effect of DL- α -difluoromethylornithine on HTC cell growth (7). Thus, addition of 1,3,6-triaminohexane to polyamine-depleted HTC cells completely reverses growth inhibition without affecting the level of decarboxylated S-adenosylmethionine (7).

The polyamines have been implicated in DNA, RNA and protein synthesis. Many of the physiological functions ascribed to the polyamines have been deduced by studying the metabolic consequences of cellular polyamine depletion. This has been possible by the development of inhibitors that specifically interfere with the various steps in the polyamine biosynthetic pathway. Using DL- α -difluoromethylornithine it has recently been demonstrated that polyamine depletion causes a dramatic increase in the intracellular level of decarboxylated S-adenosylmethionine. In the present study we have shown that the ribonucleotide pools of polyamine-depleted cells are increased as well. Obviously, the effect of polyamine depletion on a number of polyamine-related metabolites needs to be further investigated. It is equally important to consider the possible involvement of decarboxylated S-adenosylmethionine and adenine nucleotides in the inhibition of cellular processes that are presently believed to be exclusively the result of polyamine deficiency.

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TABLE 1. EFFECT OF DL- α -DIFLUOROMETHYLORNITHINE TREATMENT ON THE POLYAMINE, S-ADENOSYLMETHIONINE AND DECARBOXYLATED S-ADENOSYLMETHIONINE CONTENTS OF EHRlich ASCITES TUMOR CELLS

Treatment group ^a	S-Adenosylmethionine	decarboxylated S-Adenosylmethionine	Putrescine	Spermidine	Spermine
	(nmol/10 ⁶ cells)				
Control	0.11 (118) ^b	< 0.0002	0.16 (0)	1.29 (2)	0.88 (88)
DFMO	0.13	0.35	N.D.	0.02	0.77

^aCells were seeded at a density of 1.00×10^5 cells/ml and cultured for 72 hr in the absence or presence of 5 mM DL- α -difluoromethylornithine (DFMO).

^bEffect of DFMO treatment in % of untreated control.

N.D. = Nondetectable.

TABLE 2. EFFECT OF DL- α -DIFLUOROMETHYLORNITHINE TREATMENT ON THE ADENINE NUCLEOTIDE CONTENTS OF EHRlich ASCITES TUMOR CELLS

Treatment group ^a	Cell density (x10 ⁶ /ml)	Total protein (ug/10 ⁶ cells)	AMP	ADP	ATP	Σ A	Energy charge
			(nmol/10 ⁶ cells)				
Control	1.54 ± 0.14 (37) ^c	114 ± 14 (128)	0.16±0.06 (169)	0.36±0.10 (194)	1.35±0.38 (256)	1.86±0.31 (235)	0.82±0.06 (105)
DFMO	0.57 ± 0.12 p<0.005 ^d	146 ± 18 p<0.005	0.27±0.16 p>0.1	0.70±0.38 p<0.05	3.46±0.64 p<0.01	4.38±1.22 p<0.01	0.86±0.04 p<0.05

^aCells were seeded at a density of 1.00×10^5 cells/ml and cultured for 72 h in the presence or absence of 5 mM DL- α -difluoromethylornithine (DFMO).

^bMean $\pm$ S.D. (n = 4).

^cEffect of DFMO treatment in % of untreated control.

^dp-value as calculated with Student's one-tailed t-test.

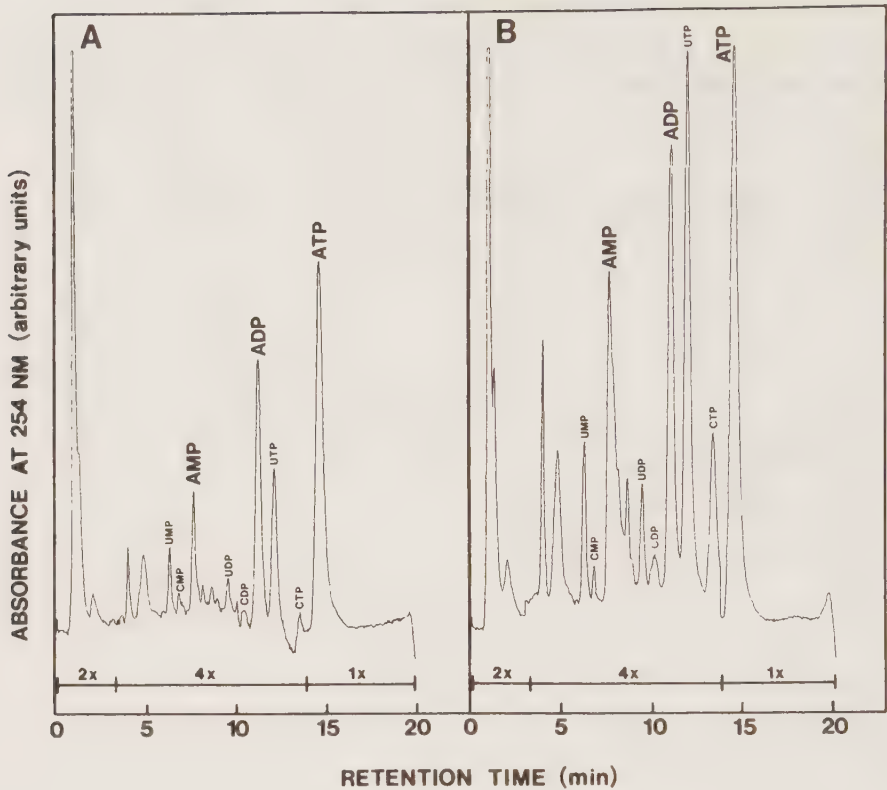


FIGURE 1. Effect of DL- α -difluoromethylornithine treatment on the ribonucleotide content of Ehrlich ascites tumor cells. The cells were grown for 72 h (A) in the absence (control) or (B) in the presence of 5 mM DL- α -difluoromethylornithine. The ribonucleotides were separated by high-performance liquid chromatography at room temperature on an anion exchanger and were detected by their absorbance at 254 nm. The amount of cell extract injected was the equivalent of 7.41×10^5 and 5.65×10^5 control and DL- α -difluoromethylornithine-treated cells, respectively - each cell number corresponding to 70.0 μ g of total cell protein. Parts of the chromatogram have been subjected to y-axis expansion by 2-4-fold, as indicated.

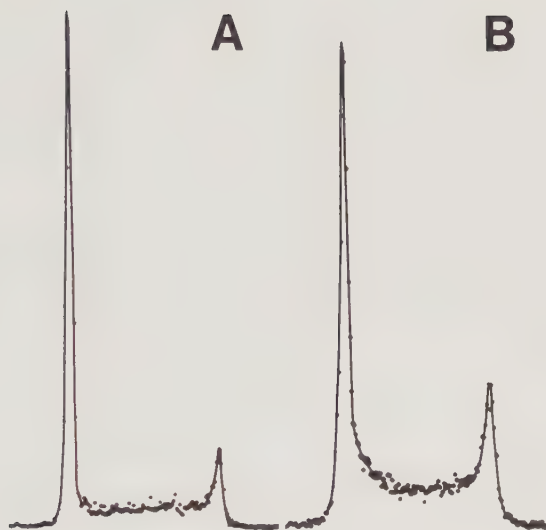


FIGURE 2. Effect of DL- α -difluoromethylornithine treatment on the cell cycle distribution of Ehrlich ascites tumor cells. The cells were grown for 72 h (A) in the absence (control) or (B) in the presence of 5 mM DL- α -difluoromethylornithine. The DNA distributions were determined by flow cytometric analysis. The y- and x-axes represent number of cell nuclei and nuclear DNA content, respectively, and are scaled arbitrarily. The DNA distributions show two peaks with relative DNA contents of 1.0 and 2.0, respectively; the first peak represents cell nuclei with a 2C DNA content (G1 phase) and the second peak represents cell nuclei with a 4C DNA content (G2 phase). The region between the 2C and the 4C peaks represents cell nuclei synthesizing DNA (S phase).

V

INDUCTION OF F9 EMBRYONAL CARCINOMA CELL DIFFERENTIATION BY INHIBITION
OF POLYAMINE SYNTHESIS

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SUMMARY

α -Difluoromethylornithine (DFMO), a highly selective inhibitor of ornithine decarboxylase (ODC), induced terminal differentiation of F9 mouse embryonal carcinoma cells in culture. Differentiation was assessed by morphological criteria and by measuring plasminogen activator activity. The nature of the phenotypic changes and the fact that the cells did not synthesize α -fetoprotein, indicates that they were parietal endoderm cells.

The putrescine, spermidine and spermine content of untreated control cells increased during exponential growth and then decreased gradually. The increases in putrescine and spermidine content were prevented by DFMO treatment. In fact, the putrescine and spermidine content decreased below the limits of detection after one day of treatment. Exogenous putrescine was able to prevent the DFMO-induced differentiation, when provided within 4 days of DFMO treatment, suggesting that the effects observed were indeed caused by polyamine depletion.

The phenotypic changes induced by DFMO were similar to those induced by retinoic acid, a very potent inducer of embryonal carcinoma differentiation. Although retinoic acid can inhibit ODC activity and putrescine accumulation, this was not found to be the mechanism behind retinoic acid-induced F9 cell differentiation, because putrescine addition did not prevent the expression of the differentiated phenotype.

Undifferentiated F9 embryonal carcinoma cells exhibited a very short G1 phase, and in this respect they are similar to the cells of the preimplantation mouse embryo. In control (exponentially growing) cultures a majority of the F9 cells were in the S phase, but in DFMO-treated cultures they became arrested in the G1 phase and showed no further proliferative potential. Our kinetic data suggest that growth arrest in the G1 phase of the cell cycle preceded the differentiation of F9 embryonal carcinoma cells into parietal endoderm cells.

INTRODUCTION

Ornithine decarboxylase (ODC) is a highly regulated enzyme which catalyzes the initial and rate-limiting step in polyamine synthesis (1). Marked increases in ODC activity and cellular polyamine content are characteristically associated with rapid growth (2,3). To manipulate the polyamine biosynthetic pathway and to delineate the functional role of ODC and the polyamines in cell growth, various inhibitors have been used (4-6). α -Difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC (7), has been used in most studies, because it is highly selective, has no acute pharmacologic activity, and is essentially nontoxic (8,9). DFMO treatment rapidly depletes the putrescine and spermidine content and reduces the rate of cell growth and division (4,5,9-11). DNA synthesis and cytokinesis seem to be the events most sensitive to polyamine limitation (10,12,13). This conclusion is supported by experiments with polyamine-auxotrophic cells (14,15).

Marked increases in ODC activity and cellular polyamine content have also been observed in association with cell differentiation (10). In many experimental models of differentiation, polyamine depletion inhibits the expression of the differentiated phenotype. This is true for chemically induced Friend erythroleukemia cells (16) and hormone-induced mammary gland (17), chondrocytes (18), L6 myoblasts (19) and 3T3 fibroblasts (20). Provision of exogenous polyamines replenishes their intracellular levels and reverses the antidifferentiative effects (16-20). It is thus apparent that the expression of the differentiated phenotype is critically dependent on continued polyamine synthesis and/or the presence of sufficient polyamine pools in the cells.

At variance with these data, we recently showed that DFMO treatment may instead induce cell differentiation (21). Thus, F9 and PCC3 mouse embryonal carcinoma cells differentiated into endoderm-like cells as a result of DFMO treatment. This finding has recently been verified using another embryonal carcinoma cell line (22). Moreover, DFMO has been found to induce morphological and functional differentiation of neuroblastoma (23) and melanoma (24) cells in culture.

In the present paper we describe the changes in polyamine content during DFMO-induced F9 cell differentiation, and analyze in greater detail the morphological changes. Biochemical markers of

differentiation (plasminogen activator and α -fetoprotein) are used to determine the nature of the differentiated cells. To establish whether the changes observed are due to polyamine depletion and whether the differentiated cells have any proliferative potential, we attempt to prevent and reverse these effects by addition of putrescine. Finally, the cell kinetic effects of DFMO treatment are analyzed in order to assess whether the differentiation of F9 cells is preceded by growth arrest in the G1 phase of the cell cycle.

MATERIALS AND METHODS

Cell line. F9 embryonal carcinoma cells were kindly provided by Dr. Nils R. Ringertz (Karolinska Institutet, Stockholm, Sweden). The F9 cell line was initially isolated from embryoid bodies of the transplantable tumor OTT 6050-970 (25), a pluripotent teratoma, which originated from the grafting of a 6-day male mouse embryo to the testis of a strain 129 mouse (26). F9 cells have been designated nullipotent because they do not form the differentiated tissues diagnostic of teratocarcinomas, when propagated in the mouse as a solid subcutaneous tumor.

Culture conditions. The F9 cells were grown in Dulbecco's Modified Eagle Medium containing 20% fetal calf serum, 1 mM pyruvate, serine, proline and non-essential amino acids, 30 μ M adenosine, guanosine, cytidine and uridine, 10 μ M thymidine and antibiotics (50 IU/ml penicillin and 50 μ g/ml streptomycin). The cells were plated in gelatinized petri dishes and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. In order to retain the undifferentiated, malignant state characteristic for embryonal carcinoma cells, the cells were thoroughly dissociated with a 0.25% trypsin solution (containing 0.02% EDTA) and subcultured every second day. In the exponential phase of growth they undergo only very limited spontaneous differentiation.

Experimental treatment of cells. For the experiments, 2.5×10^5 cells were plated in 15 ml of medium (in 90 mm petri dishes) in the absence or presence of 1 μ M retinoic acid or 5 mM DFMO. Retinoic acid was always freshly prepared as a 1 mM stock solution in absolute ethanol and was

added directly to the culture medium to give a final concentration of 1 μ M. The final concentration of ethanol in the medium was less than 0.1% and did not affect growth or differentiation of the cells. All procedures involving retinoic acid were carried out under dim light. Before addition to the culture, DFM0 was dissolved in phosphate-buffered saline (PBS) at a 500 mM concentration, the pH adjusted to 7.4 and the solution sterile filtered. The medium was changed every second day. Cells grown in the presence of retinoic acid for two days received retinoic acid-free medium at all medium changes. DFM0-treated cells, however, received a medium containing 5 mM DFM0 at all medium changes.

Polyamine analysis. Cells intended for polyamine analysis were pelleted by centrifugation at 1000xg for 10 min at 4°C. The pellets were stored at -20°C until analysis. The cell pellets were disrupted by sonication in ice-cold 0.2M perchloric acid and the homogenates were kept on ice for 1 hr. The polyamine content of the acid-soluble cell extracts was determined by thin-layer chromatography (27), essentially as described by Heby et al. (28).

Flow cytometric analysis. Cells intended for flow cytometric analysis were pelleted at 500xg for 5 min at room temperature. The medium was decanted and the cells were resuspended in the small volume of remaining medium. Single-cell suspensions ($1-2 \times 10^6$ cells) were fixed in 1 ml of absolute ethanol (-20°C) and were stored at 4°C until analysis. Fixed cells were treated with RNase and pepsin (29). The resulting nuclei were passed through a nylon mesh with a pore size of 50 μ m, and finally stained with propidium iodide (29). Propidium iodide-stained cells, were analyzed on a Cytofluorograph System 50-H (Ortho Instruments, Westwood, MA, USA) equipped with a 4 W argon-ion laser (Model 95, Lexel Corp., Palo Alto, CA, USA). Since most mitotic cells lack a nuclear membrane, they are likely to be destroyed by pepsin (30). Therefore, they make no contribution to the 4C DNA peak. The nuclei of late-telophase cells may separate and appear in the first (G1) peak of the histogram (30). Pepsin treatment is also likely to disperse the nuclei of multinucleate cells, which may become more frequent during the course of polyamine starvation. The percentages of cells in each phase of the cell cycle were derived from the DNA distributions essentially as described by Dean (31).

Plasminogen activator analysis. The growth medium was aspirated and the cells intended for plasminogen activator analysis were washed three times with ice-cold PBS. Aliquots of 0.05% Triton X-100 in PBS were added to the petri dishes, which were kept on ice. The cells were then disrupted by repeated pipetting for 10 min. The cell homogenates were stored in test tubes at -20°C until analysis. The plasminogen activator analysis was performed essentially as described by Saksela (32). Non-fat dry milk (the source of casein) was dissolved as a 15% stock solution in PBS, heated at 95°C for 30 min, centrifuged at $1000\times g$ for 10 min and stored at -20°C . Sodium azide (added to prevent microbial growth) was made up as a 2% stock solution in PBS. Plasminogen was dissolved as a $100\text{ }\mu\text{g/ml}$ stock solution in distilled water and stored at -20°C . A human urokinase preparation was used as standard. Dilutions were made in PBS to give a final concentration of 0.001-50 Plough units/ml (33). The standards were stored at -20°C . A 1.2% agarose solution was prepared in 0.1M Tris-HCl buffer, pH 8.0, by boiling for 10 min. The agarose solution (19 ml) was cooled to 50°C before the addition of casein (0.8 ml), plasminogen (0.34 ml) and sodium azide (0.5 ml). Control gels received 0.34 ml of distilled water instead of plasminogen. Each mixture was rapidly poured into the space between two glass plates, separated by a 1 mm plastic frame and held together with clips. An agarose gel support film (Gel Bond film, Bio Products, Rockland, Maine, USA) had been previously adhered to one of the glass plates. After cooling, the glass plate that was not supporting the film was slid away and sample wells with a diameter of 3.5 mm were punched out in the gel. Ten μl of 3% sodium dodecyl sulphate was added to each 100 μl sample and standard. Ten- μl aliquots of these mixtures were applied in the wells. The gels were incubated at 37°C for 72 hr and the diameters of the clear circular areas, in which the casein had been degraded, were measured (Fig. 4).

Nomarski differential interference contrast microscopy. For morphological studies 2.5×10^5 cells were plated into 15 ml of medium, and cultured in the absence or presence of $1\text{ }\mu\text{M}$ retinoic acid or 5 mM DFMO in 90 mm petri dishes containing small gelatinized glass slides. The cells were cultured as described above. At various times after plating, glass slides were removed, rinsed in PBS and placed up side down on regular size glass slides. The cells were photographed with Nomarski differential interference contrast optics (Olympus BH-2 System Microscope).

Reversal of DFMO-induced differentiation by putrescine. For this study 8×10^4 cells were plated into 5 ml of medium, and cultured in the absence or presence of $1 \mu\text{M}$ retinoic acid or 5 mM DFMO, in 50 mm petri dishes. The growth medium was changed every second day. Cells grown in the presence of retinoic acid for two days received retinoic acid-free medium at all medium changes. At 0, 2, 4, 6, 8 or 10 days after plating, the cell cultures were supplemented with $50 \mu\text{M}$ putrescine. Before addition to the cultures, putrescine was dissolved in PBS at a 5 mM concentration, the pH adjusted to 7.4 and the solution sterile filtered. At each change of medium those supplements (DFMO and/or putrescine) were added, that had been present prior to the change. The cells were observed and photographed by phase contrast microscopy on a daily basis for 19 days.

Chemicals. Agarose (Type B) and human urokinase were purchased from Calbiochem-Behring (San Diego, CA, USA), human plasminogen from Kabi Vitrum (Stockholm, Sweden), the nucleotides, pyruvate, serine, proline and putrescine from Fluka (Buchs, Switzerland), all-*trans*-retinoic acid from Sigma Chemical Co. (St. Louis, MO, USA) and growth medium components from Gibco Europe (Paisley, Scotland).

DL- α -difluoromethylornithine monohydrochloride monohydrate (DFMO) was generously donated by Centre de Recherche Merrell International (Strasbourg, France).

RESULTS

Figure 1 shows the effects of $1 \mu\text{M}$ retinoic acid and 5 mM DFMO on growth and polyamine content of F9 embryonal carcinoma cells. Untreated control cells exhibited exponential growth for about 3 days after plating (Fig. 1A). The population doubling time was approximately 9 hr during this period. Retinoic acid and DFMO treatment resulted in growth inhibition, that was apparent by 2 days after seeding. The DFMO-treated cells completely stopped growing at this point and the cell number remained constant for the remainder of the 8 day experimental period. At least a certain fraction of the retinoic

acid-treated cells resumed growth after the removal of retinoic acid from the growth medium (Day 2) and exhibited exponential growth from Day 3 to 8. The population doubling time of these cells, however, was three times longer than that of untreated control cells in exponential growth.

The putrescine, spermidine and spermine content of untreated F9 cells increased after plating and reached maximum levels by Day 1 (Fig. 1B-D), i.e. at the time of most rapid proliferation (Fig. 1A). In fact, during the entire period of exponential growth the cells exhibited elevated polyamine levels.

During the first 2 days after plating, retinoic acid-treated F9 cells exhibited patterns of changes in polyamine content that were basically similar to those of untreated control cells (Fig. 1B-D), even though growth was markedly reduced (Fig. 1A). Upon removal of retinoic acid from the growth medium (Day 2) there was a marked increase in putrescine, spermidine and spermine content. Maximum levels were reached by Day 4, i.e. during the early phase of exponential growth.

DFMO treatment not only prevented the increases in cellular putrescine and spermidine content but decreased their basal levels (Fig. 1B, C). In fact, no putrescine or spermidine was detectable from Day 1 through 8. In DFMO-treated cells the spermine content increased initially and then returned to the basal level (Fig. 1D). By and large, the spermine content remained at the basal level throughout the period of DFMO-induced growth arrest, i.e. from Day 2 to 8.

Figure 2 shows the effects of retinoic acid and DFMO on the cell cycle distribution of F9 cells. During the exponential phase of growth (Day 0-3) an average of 27.7% of the untreated control cells were in G1, 56.4% were in S, and 15.9% were in G2 (Fig. 2A). Four to 5 days after plating, when the growth rate declined (Fig. 1A), there was a slight decrease in the S and G2 phase fractions (Fig. 2A). Assuming a growth fraction of 1.0 and a mitotic duration of 1.0 hr for untreated control cells in exponential growth, one can estimate the durations of the other cell cycle phases by using the average phase fractions obtained for exponentially growing cells. The estimated G1, S and G2 phase durations were 2.2, 4.4 and 1.4 hr, respectively.

In retinoic acid- and DFMO-treated cells, there was a decrease in the S and G2 phase fractions already one day after plating (Fig. 2).

This was followed by a further decrease on Day 2. In retinoic acid-treated

as well as in DFMO-treated cultures, the cell cycle distribution did not change to any appreciable extent from Day 2 to 6, but there was a certain difference between their respective patterns. Thus, retinoic acid-treated cells (Fig. 2B) consistently exhibited a larger G2 phase fraction than did DFMO-treated cells (Fig. 2C). This may be due to the fact that the retinoic acid-treated cells were still cycling, whereas the DFMO-treated cells were not (Fig. 1A). In the latter, growth-arrested cultures about 68% of the cells were in G1, 28% were in S, and 4% were in G2 (Fig. 2C).

Figure 3 shows the effects of retinoic acid and DFMO on the morphology of F9 cells. Untreated control cells grew as tightly packed colonies, characteristic of embryonal carcinoma cells (Fig. 3A, D). The cell population appeared predominantly homogenous during the first 3 days of growth, and consisted of small cells with a round shape and a large nucleus:cytoplasm ratio (Fig. 3A, D). Each nucleus contained 1-2 prominent nucleoli. By Day 5 the cultures were overcrowded and the cells began to detach from the substratum (not shown). Occasionally, patches of cells with different morphology were observed (21). Large numbers of mitotic figures, consistent with the rapid proliferation of the F9 cells, were observed in the control cultures.

Upon addition of retinoic acid or DFMO to F9 cell cultures, a pronounced morphological change occurred during the next several days (Fig. 3). Within a day, the cells moved apart and the colonies became much less compact. Then the cellular morphology changed gradually to triangular, flat cells with perinuclear granules (Fig. 3). The appearance of these cells closely resembled that of endoderm cells. The differentiating cells greatly increased in size and their nucleus:cytoplasm ratio decreased. Terminally differentiated giant cells appeared after a period of 8 to 13 days (Fig. 3I, J and Fig. 6E, H, K, L).

The plasminogen activator activity of F9 cells induced to differentiate by retinoic acid and DFMO treatment, was evaluated by a radial caseinolysis assay in agarose gels (Fig. 4). Such analyses may yield erroneous results because plasminogen activator inhibitors are present in most sera used in cell culture media (32). Therefore, sodium dodecyl sulphate was added to samples and standards (to yield a final concentration of 0.3%) in order to dissociate any plasminogen activator-inhibitor complexes present. Since the inactivation of plasminogen activator exerted by sodium dodecyl sulphate is reversible, the protease activity

is recovered upon radial dilution of the detergent. The low background proteolysis observed in gels lacking plasminogen (Fig. 4B, D, F) is probably due to the presence of serum- and/or cell-derived plasmin and/or other proteases capable of degrading casein.

In untreated control cells, the plasminogen activator activity did not appear to exceed the background (Fig. 4A, B). However, since the lysis zones were not well demarcated in the 3- to 5-day samples, we cannot exclude the possibility that they might have concealed a low activity. In retinoic acid-treated cells, the plasminogen activator activity exceeded the background already after one day. Notably, the diameters of the lysis zones were similar from Days 2 to 8 despite the fact that the 10 μ l cell homogenates applied in the wells contained an increasing number of cells. In DFMO-treated cells, the plasminogen activator activity did not exceed the background during the first 2 days (Fig. 4E). From Day 3 and on, however, it significantly exceeded background activity. The linear relationship that was obtained when the diameters of the caseinolysis zones of the urokinase standards were plotted against the logarithm of the urokinase concentration (not shown), was used to quantitate plasminogen activator activity. The detection limit of the assay was 0.001 Ploug units/ml.

Figure 5 shows the plasminogen activator activity of F9 cells induced to differentiate by retinoic acid and DFMO treatment. The data points were derived from the radial caseinolysis assay shown in Figure 4. In retinoic acid-treated cells, the plasminogen activator activity increased to a maximum level by Day 3, and then decreased as the cells resumed to proliferate (Fig. 1A). In DFMO-treated cells, there was no significant plasminogen activator activity during the first 2 days of treatment. The activity then increased to a maximum by Day 5 and remained elevated until the end of the experiment. Notably, the maximum plasminogen activator activity induced by retinoic acid treatment was 7-fold higher than that induced by DFMO treatment.

Figure 6 shows an experiment in which putrescine was administered to F9 cell cultures at various times after the addition of DFMO, in an attempt to prevent the expression of a differentiated phenotype. An inhibitory effect on DFMO-induced differentiation, as evaluated by morphological criteria, was obtained only when putrescine was added within 4 days of DFMO treatment (Fig. 6C, F). Virtually no effect on DFMO-induced differentiation was seen if putrescine was added at a later

time, i.e. the morphology did not revert back to that of embryonal carcinoma cells (Fig. 6I, L).

DISCUSSION

The results of studies that have attempted to determine the role of polyamines in differentiation may be divided into three groups, based on the effects of DFMO treatment on the expression of the differentiated phenotype. 1) Induction of differentiation of a variety of normal cells by chemicals or hormones, results in stimulation of the ODC activity and an increase in cellular polyamine content (16-20). When the increase in ODC activity is inhibited by DFMO treatment, the cells become depleted of their putrescine and spermidine, and differentiation does not occur. This antidifferentiative effect of DFMO may be prevented or reversed by the addition of polyamines. 2) Induction of differentiation of human promyelocytic leukemia (HL-60) cells by retinoic acid and phorbol esters, also results in stimulation of polyamine synthesis, but differentiation is not inhibited by DFMO treatment (21, 34, 35). Thus, the increase in ODC activity and polyamine content induced by retinoic acid or phorbol esters does not appear to be necessary for the differentiation of these cells. 3) Inhibition of the ODC activity by DFMO induces differentiation of some highly malignant cell types; embryonal carcinoma cells (21, 22), neuroblastoma cells (23) and melanoma cells (24). Similarly, methylglyoxal-bis(guanylhydrazone), a potent inhibitor of S-adenosylmethionine decarboxylase apparently induces differentiation of keratinocytes by interfering with polyamine synthesis (36).

In this paper we have extended our study of the DFMO-induced differentiation of F9 embryonal carcinoma cells. Embryonal carcinoma cells are highly malignant and constitute the proliferating stem cells of tumors known as teratocarcinomas. Initially, the F9 embryonal carcinoma cells were considered "nullipotent", because they exhibited a very low frequency of spontaneous differentiation under normal culture conditions. However, Strickland and Mahdavi (37) recently demonstrated that physiological concentrations of retinoic acid can induce F9 cell differentiation *in vitro*. The differentiated phenotype was distinguished from undifferentiated embryonal carcinoma cells by altered cellular morphology and biochemical properties. The results presented here demonstrate that the morphological changes induced by DFMO treatment

are similar to those induced by retinoic acid. The appearance of the differentiated cells is clearly similar to that of endoderm cells.

To corroborate the interpretation made on the basis of morphological criteria, we used a biochemical marker, plasminogen activator, which has been reported to distinguish endoderm cells from embryonal carcinoma cells (37). Primitive endoderm cells and the more differentiated parietal endoderm cells synthesize and secrete plasminogen activator, whereas visceral endoderm does not (38). Untreated control cells were not found to contain significant amounts of plasminogen activator. Addition of DFMO to the cultures, however, increased the plasminogen activator content, but not nearly to the level reached in retinoic acid-treated cells. Moreover, the increase was delayed compared to that induced by retinoic acid. Using morphology and plasminogen activator activity as criteria for identification, the cell type generated upon treatment of F9 cells with DFMO was considered similar to parietal endoderm cells. This contention was also supported by the fact that these cells did not synthesize α -fetoprotein (not shown). Thus, parietal endoderm cells do not synthesize α -fetoprotein whereas visceral endoderm cells do (39, 40).

In untreated control cultures the cellular levels of putrescine, spermidine and spermine increased during exponential growth, and then decreased gradually. DFMO treatment not only prevented the increases in putrescine and spermidine content but decreased their levels below the limits of detection, already after one day of treatment. To determine whether DFMO-induced F9 cell differentiation was indeed due to this polyamine depletion, putrescine was added at various times of DFMO treatment. Provided that putrescine was administered within 4 days of DFMO treatment, there was no induction of differentiation, as determined by morphological criteria. This reversal by putrescine cannot be due to competition for DFMO uptake as it has been demonstrated that the uptake is not affected by polyamines (41, 42). These results suggest that alterations in the levels of cellular polyamines play a role in embryonal carcinoma cell differentiation.

Several reports indicate that retinoic acid can inhibit ODC activity (43, 44). However, the fact that putrescine administration did not prevent retinoic acid-induced differentiation (not shown) demonstrates that inhibition of ODC activity is not the means by which retinoic acid mediates the induction of differentiation.

to parietal endoderm cells.

Undifferentiated F9 cells exhibited a very short G1 phase (2.2 hr), as was also shown by others (45). In this respect they are similar to the cells of the preimplantation mouse embryo (46). In exponentially growing control cultures a majority of the cells were in the S phase. As a result of DFMO treatment, however, the cells became arrested in G1. After a 3 to 4-day period of polyamine depletion these cells were in a state of terminal differentiation. Upon addition of putrescine they showed no further proliferative potential. Kinetic analyses suggest that growth arrest in the G1 phase of the cell cycle (as a result of retinoic acid or DFMO treatment) preceded the differentiation of F9 embryonal carcinoma cells into parietal endoderm cells.

The fact that DFMO treatment inhibits growth and induces terminal differentiation of some highly malignant tumor types, yet exhibit minimal toxicity, suggests that this compound may have clinical utility (alone or in combination with other chemotherapeutic agents) in the treatment of proliferative disorders in general and differentiation-responsive tumors in particular.

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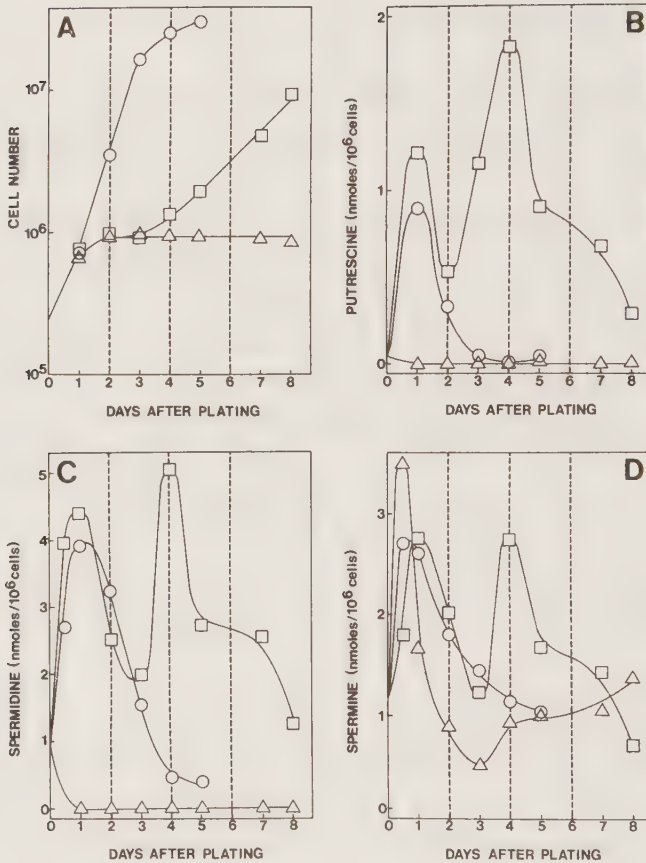


Figure 1. Effects of retinoic acid and DFMO on F9 embryonal carcinoma cell growth (A) and polyamine content (B-D). Retinoic acid ($1 \mu\text{M}$) and DFMO (5 mM) were added at the time of plating. The growth medium was changed every second day. Cells grown in the presence of retinoic acid for two days received retinoic acid-free medium at all medium changes. DFMO-treated cells, however, received a medium supplemented with 5 mM DFMO at all medium changes. $\circ$, untreated control cells; $\square$, retinoic acid-treated cells; $\triangle$, DFMO-treated cells.

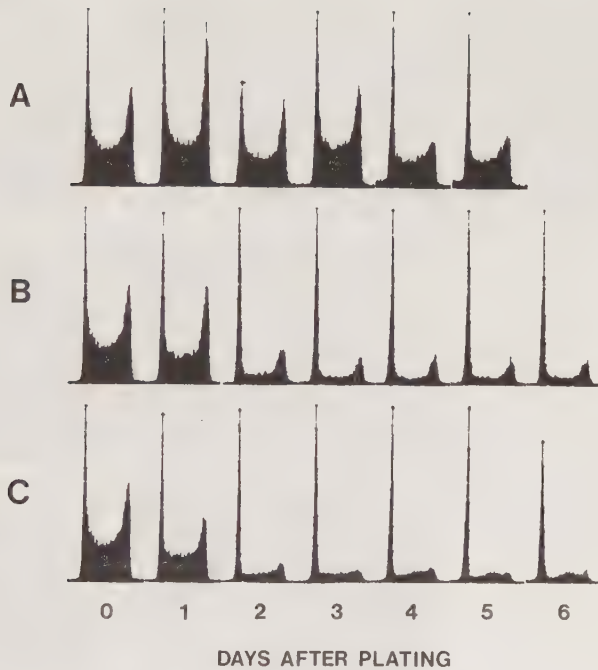


Figure 2. Effects of retinoic acid and DFMO on the cell cycle distribution of F9 embryonal carcinoma cells. Treatment with retinoic acid ($1 \mu\text{M}$) and DFMO (5 mM) were as described in Fig. 1. The DNA distributions were determined by flow cytometric analysis. The y- and x-axes of the individual DNA histograms represent number of cell nuclei and nuclear DNA content, respectively, and are scaled arbitrarily. The individual DNA distributions show two peaks with relative DNA contents of 1.0 and 2.0, respectively; the first peak represents cell nuclei with a 2C DNA content (G1 phase) and the second peak represents cell nuclei with a 4C DNA content (G2 phase). The region between the 2C and the 4C peaks represent cell nuclei synthesizing DNA (S phase). Panel A. Untreated control cells; Panel B. Retinoic acid-treated cells; Panel C. DFMO-treated cells.

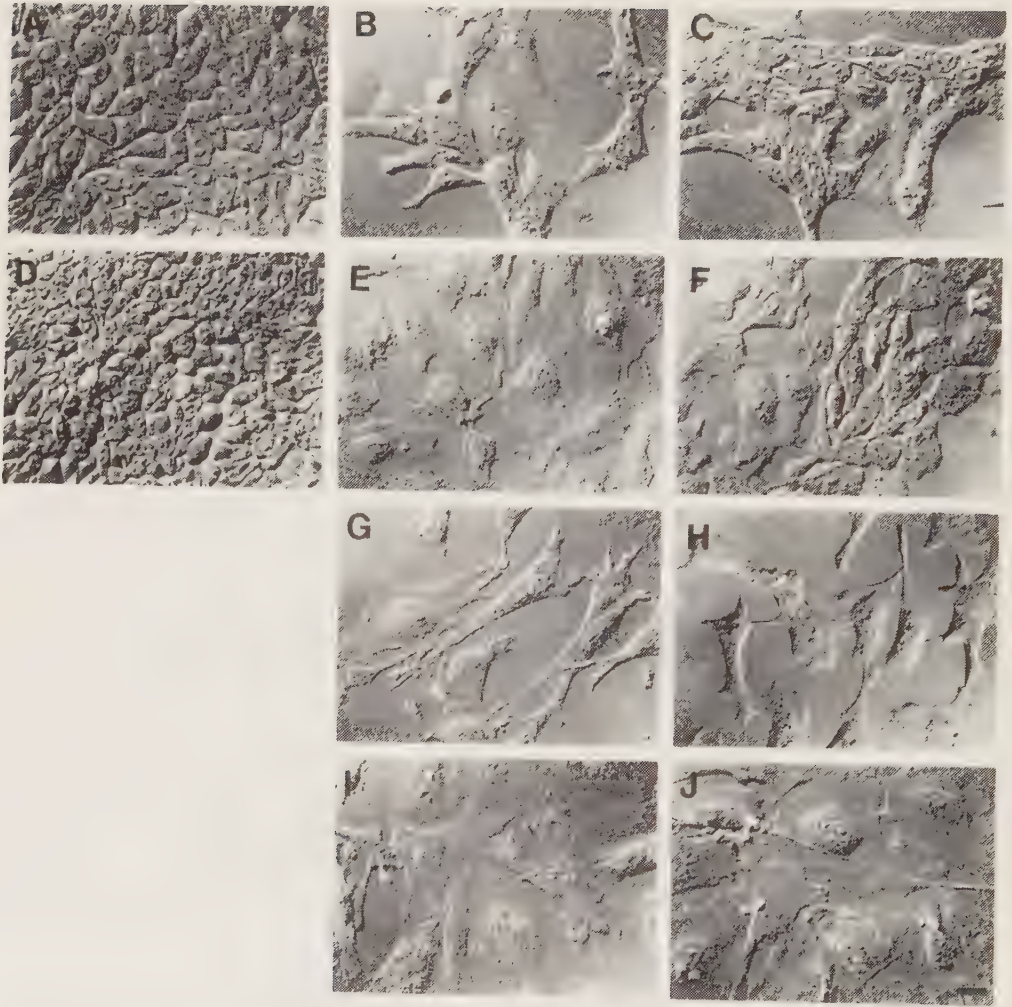


Figure 3. Effects of retinoic acid and DFMO on the morphology of F9 embryonal carcinoma cells. Treatment with retinoic acid ($1 \mu\text{M}$) and DFMO (5 mM) were as described in Fig. 1. Untreated control cells (A, D), retinoic acid-treated cells (B, E, G, I) and DFMO-treated cells (C, F, H, J) were photographed at 2 (A-C), 3 (D-F), 5 (G, H) and 8 (I, J) days after plating. Nomarski differential interference contrast micrographs. Olympus BH-2 System Microscope. S plan 40X achromatic objective. Bar, $20 \mu\text{m}$.

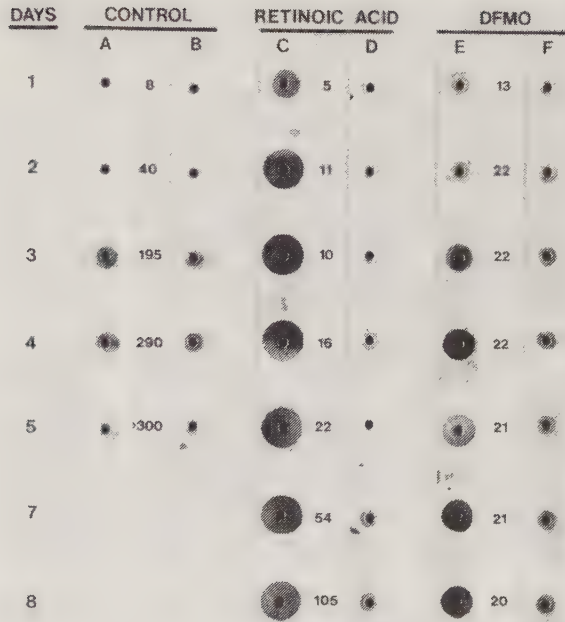


Figure 4. Effects of retinoic acid and DFMO on the plasminogen activator activity in F9 embryonal carcinoma cells as evaluated by a radial caseinolysis assay in agarose gels. Treatment with retinoic acid (1 μ M) and DFMO (5 mM) were as described in Fig. 1. (A, C, E) Gels containing plasminogen. (B, D, F) Control gels lacking plasminogen. The figures specified indicate the number of cells (thousands) contained in each 10- μ l homogenate applied in the various wells. The agarose gels were photographed against a black background.

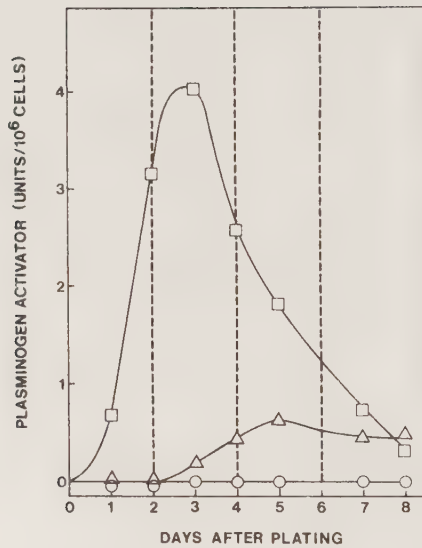


Figure 5. Effects of retinoic acid and DFMO on the plasminogen activator activity in F9 embryonal carcinoma cells. Treatment with retinoic acid (1 μ M) and DFMO (5 mM) were as described in Fig. 1. ○, untreated control cells; □, retinoic acid-treated cells; △, DFMO-treated cells. The data points were calculated from those obtained in Fig. 4.

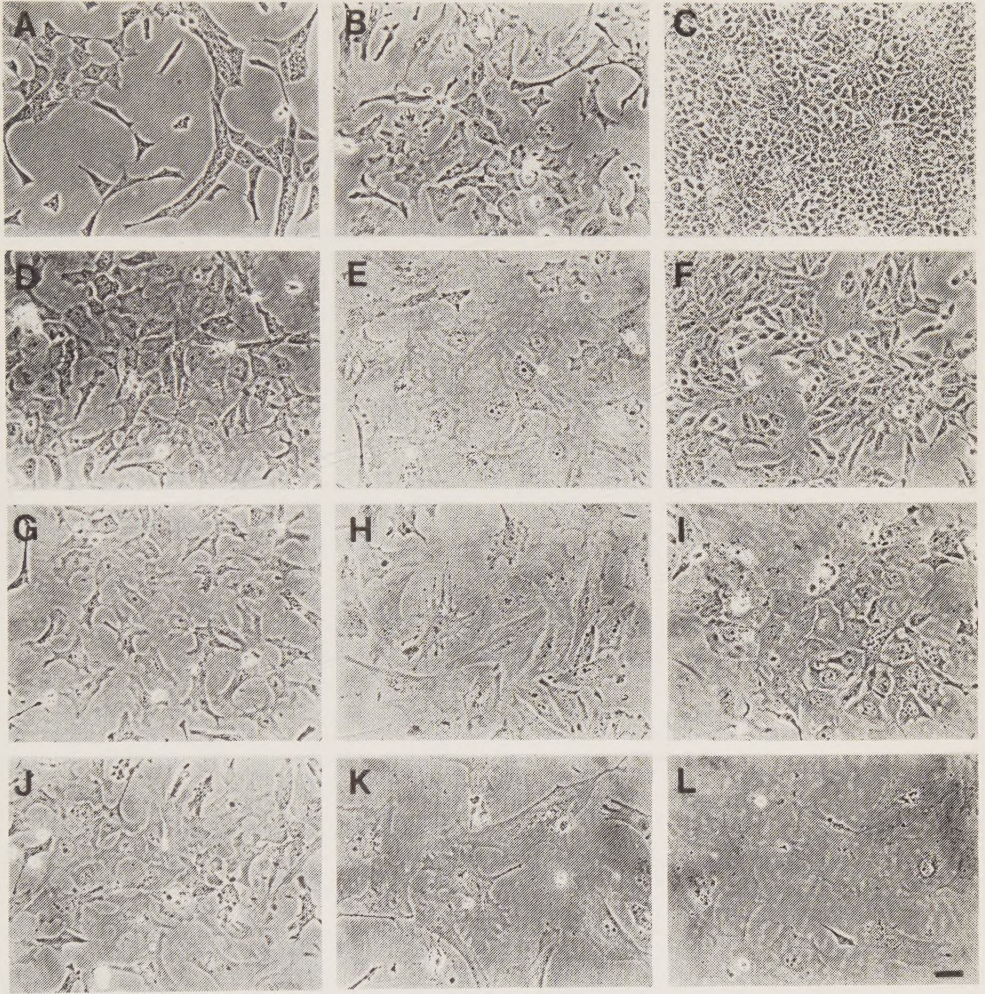


Figure 6. Analysis of the capacity of putrescine to reverse DFMO-induced F9 embryonal carcinoma cell differentiation. DFMO (5 mM) was added at the time of plating. At 2, 4, 6 or 8 days after plating, the cell cultures, which all contained DFMO, were supplemented with 50 μ M putrescine. At each change of medium (every second day) those supplements (DFMO and/or putrescine) were added, that had been present prior to the change. (A, D, G, J) Cell morphology at the time of putrescine supplementation, i.e. days 2, 4, 6 and 8, respectively. (C, F, I, L) Cell morphology after 5 days of putrescine treatment, i.e. days 7, 9, 11 and 13, respectively. The morphology of cells that were not supplemented with putrescine are shown for comparison; B, E, H and K correspond to days 7, 9, 11 and 13, respectively. Phase contrast micrographs. Bar, 40 μ m.

